Novel Biomarkers in the Diagnosis and Pathogenesis of Immunobullous Disorders

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King's College London

Novel Biomarkers in the Diagnosis and Pathogenesis of Immunobullous Disorders

A thesis submitted for the degree of Doctor of Philosophy

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2015

Abstract

Mucous membrane pemphigoid (MMP) and pemphigus vulgaris (PV) are uncommon mucocutaneous immunobullous disorders associated with significant morbidity. The aims were to: 1) Investigate the potential of saliva for diagnosis and disease-monitoring 2) Utilize serum and salivary biomarkers in the analysis of disease severity and therapeutic responses 3) Correlate phenotypic subgroups with target antigens to potentially explain the spectrum of clinical presentation and disease severity in MMP.

Methodology: Matched serum and whole saliva samples were taken from a total of 100 MMP patients, 26 PV patients, 50 healthy controls (HC) and 16 Lichen planus (LP) patients. All LP and PV patients, 40 MMP and 6 HC provided parotid saliva. Reactivity with the NC16a epitope on bullous pemphigoid antigen 2 (BP180), the alpha 6 beta 4 integrin (α6β4) and desmoglein 3 (Dsg3) was determined by Enzyme-linked immunosorbent assays (ELISA). Disease severity was assessed using the Guy's Oral Disease Severity Score.

Results: In MMP, IgG and/or IgA antibody to BP180-NC16a (44%) in whole saliva showed a similar sensitivity to serum (48%). In parotid saliva IgA to BP180-NC16a was positive in 45% and was also secretory component positive ($r = 0.9141$). A change in serum antibodies (IgG and IgA) to BP180-NC16a was significantly related to a change in severity scores ($p = 0.048$ and 0.033, respectively). A positive association was found between 1) the presence of serum IgG to BP180-NC16a and the presence of lesions in multisite disease ($p = 0.003$) and 2) the presence of IgA to BP180-NC16a in whole saliva and the presence of oral and ocular lesions ($p = 0.016$). IgG antibody to α6β4 integrin was positive in 36% of serum and 18% of saliva.

Regarding PV, anti-Dsg3 IgG antibody was positive in serum (74%) and whole saliva (61%) with a positive correlation ($r = 0.9044$). Serum IgA anti-Dsg3 antibody was positive in 61% (with a combined IgG positivity of 78% in PV patients). A significant

relationship between anti-Dsg3 IgG antibody in whole saliva and therapeutic responses was found ($p = 0.004$).

Conclusions: The results show 1) the value of salivary anti-Dsg3 IgG antibodies in monitoring PV patients and confirm the sensitivity and specificity of IgG anti-Dsg3 serum and saliva antibodies in PV diagnosis. The high positivity of serum IgA anti-Dsg3 antibodies requires further elucidation in disease pathogenesis. The salivary biomarkers, IgG and IgA antibodies to BP180-NC16a and α6β4 may be useful in the diagnosis of MMP. 2) IgG and IgA antibodies to BP180-NC16a in serum can be used for disease monitoring and therapeutic responses in MMP. 3) Clinical phenotypes did not appear to be explained by epitope or isotype specificity. However, the novel finding of locally produced antibodies to BP180-NC16a may provide further insight into the pathogenesis of the oral disease. These new findings demonstrate the value of saliva in the diagnosis and monitoring of MMP and for furthering the understanding of disease pathogenesis.

Acknowledgments

I would like to acknowledge King Abdulaziz University and the Saudi Cultural Bureau for providing me with this opportunity. I am very much grateful to my supervisor, Dr. Jane Setterfield for her constant support, dedication and advice while maintaining an amicable atmosphere. This thesis would not have come to be without her valuable input. In addition, I also recognize Professor Charles Kelly for his continuous lab assistance and persistent supervision in all aspects of this work. Professor Stephen Challacombe's rigorous meetings and efforts must be acknowledged as he created an inquisitive environment relentlessly challenging ideas and results and for his critical encouragement throughout this thesis. I would like to give my thanks to my colleagues in the department for their help providing valuable lab support, these include; Mukesh Mistry, Durdana Rahman for her advice on ELISA techniques and David Moyes for his beneficial ideas and suggestions. In addition, a special thank you to Carlo Scala who provided invaluable support for protein experiments. I extend my thanks to Stela Monk and Marta Zincola for their much appreciated aid with protein purification, ELISA and western blotting. The Oral medicine and Dermatology department staff at Guy's Hospital who kindly helped in sera collection as well as our collaborator's at Moorefields Eye Hospital. A great thank you goes to Nora Donaldson for her statistical advice.

My greatest acknowledgments go to my family; I am forever grateful for your love and support. Sincere thanks to my parents, for their steadfast care throughout. My husband Majed, without you, none of this would have been possible. To my beautiful daughters, Fatima and AlBatool, you are my constant sources of ambition.

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List of Awards and Publications

- Best presentation in the Post graduate research day (PGR) at King's College London 2013 and 2014
- Secured a grant from The friends of Guys Hospital, 2014
- Poster presentation at the British society of dental research meeting (BSODR) in Bath, UK 2013
- Oral presentation at the American association of oral medicine (AAOM) in Florida, USA 2014 (presented by Prof Challacombe)
- Review article 'Update on the clinical features, pathogenesis and investigation of immunobullous disorders' Faculty Dental Journal October 2013 volume 4 issue 4
- Abstract 'Salivary antibodies against BP180-NC16a in diagnosing and monitoring patients with mucous membrane pemphigoid' Oral Surgery Oral Medicine Oral Pathology Oral Radiology 2014 volume 117 issue 5 pages e334-e335
- Manuscript 'Salivary IgA and IgG Antibodies to BP180-NC16a as Diagnostic Biomarkers in Mucous Membrane Pemphigoid' (In press Br J Dermatol)
- Manuscript 'Serum and Salivary IgG and IgA Antibodies to Dsg 3 in Mucosal Pemphigus Vulgaris' (In press Br J Dermatol)
- Manuscript 'Serum and Salivary IgG Antibodied to A6B4 Integrin in Mucous Membrane Pemhigoid' (In Preparation)

List of Abbreviations

α6β4	Alpha 6 beta 4 integrin
aa	Amino acid
ANOVA	One-way analysis of variance
BMZ	Basement membrane zone
BP	Bullous pemphigoid
BP180	Bullous pemphigoid antigen 2
BSA	Bovine serum albumin
cDNA	Complimentary DNA
DC	Disease control
dH_2O	Distilled water
DIF	Direct immunofluorescence
DNA	Deoxy ribonucleic acid
Dsc	Desmocollin
Dsg	Desmoglein
Dsg1	Desmoglein 1
Dsg3	Desmoglein 3
E.coli	Escheria Coli
ELISA	Enzyme linked immunosorbent assay
Fc	Flowcell
GALT	Gut associated lymphoid tissue
GST	Glutathione S transferase
HC	Healthy control
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IIF	Indirect immunofluorescence
IL	Interleukin
IPTG	Isopropyl d-thioglactopyranoside

K_D	Equilibrium dissociation constant
Kda	Kilo Dalton
LB	Luria Bertani
LDS	Lithium dodecyl sulphate
LP	Lichen planus
M	Molar
Mab	Monoclonal antibody
MALT	Mucosa associated lymphoid tissue
MHC	Major histocompatibility complex
ml	Milli litre
mM	Milli molar
MMP	Mucous membrane pemphigoid
NaCl	Sodium chloride
NALT	Nasal associated lymphoid tissue
NaOH	Sodium hydroxide
$NISO_4$	Nickel sulfate
nm	Nanometer
NC16a	Non-collagenous domain 16a
OD	Optical density
PBS	Phosphate buffered saline
PF	Pemphigus foliaceus
PV	Pemphigus vulgaris
RC	Reference cell
Rpm	Revolution per minute
RU	Resonance unit
SC	Secretory component
SDS	Sodium dodecyl sulphate
SIgA	Secretory immunoglobulin A
SOC	Super optimal broth with glucose
SPR	Surface plasmon resonance

SSS	Salt split skin
T20	Tween 20 (polysorbate 20)
TGFβ	Tumour growth factor beta
Th	T helper cell
Treg	Regulatory T cell
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloric acid
µg	Microgram
µl	Microlitre

1 Introduction

1 Introduction: an overview of mucous membrane pemphigoid (MMP) and pemphigus vulgaris (PV)

This chapter provides an overview of the two diseases focused on in this thesis, mucous membrane pemphigoid (MMP) and pemphigus vulgaris (PV). The review covers the historical background, epidemiology, clinical presentation, antibody detection, the pathogenesis and the target antigens for each disease. In addition, it gives a short account on mucosal immunity and the use of saliva as a medium for biomarker detection.

Mucous membrane pemphigoid and pemphigus vulgaris are both autoimmune vesiculobullous diseases usually referred to as immunobullous disorders. In these disorders, autoantibodies target antigens in the skin and mucous membranes resulting in fragility and blistering. Vesicles (≤ 5 mm) and bullae (> 5 mm) are fluid-filled lesions forming within or just below the epidermis/epithelium. They frequently rupture to form an ulcer. MMP is characterised by sub-epithelial separation as shown in (Fig 1-1) and histologically shown in (Fig 1-2 a). PV is characterised by acantholysis or breakdown of cell attachments known as desmosomes leading to intra-epithelial separation usually forming a suprabasal cleft (Figs 1-1 and 1-2 b). In these diseases, damage to the target autoantigens results in significant morbidity and occasionally mortality.

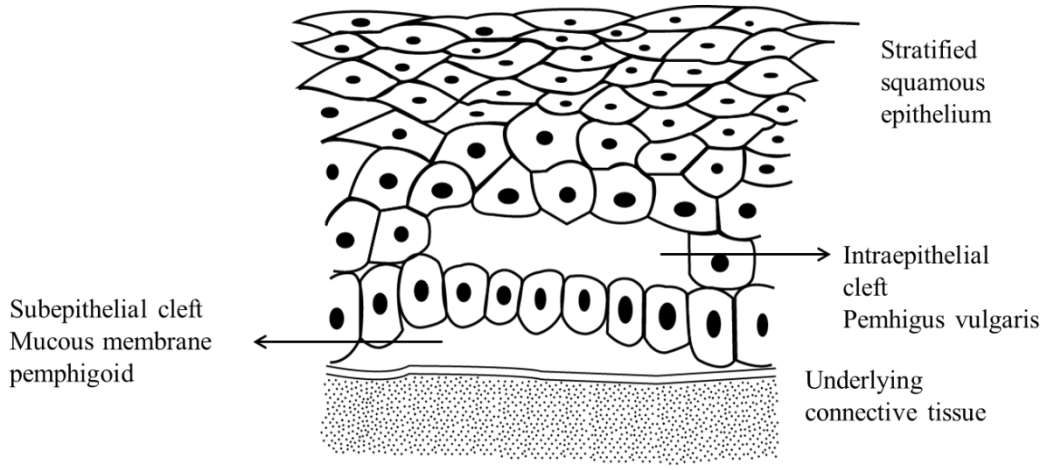
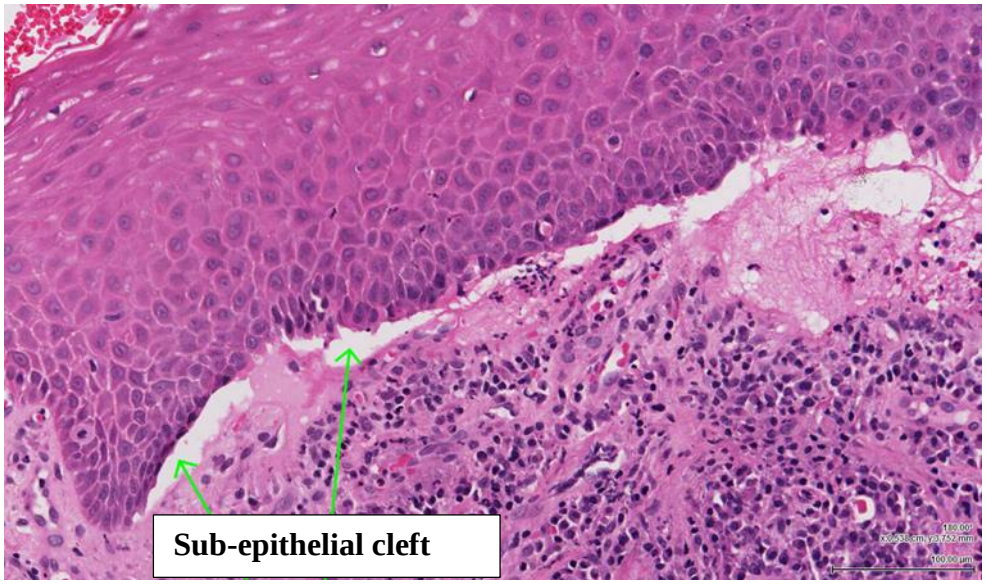


Figure 1-1 A schematic drawing showing the sites of cleavage in the epithelial level in MMP and PV

a)



b)

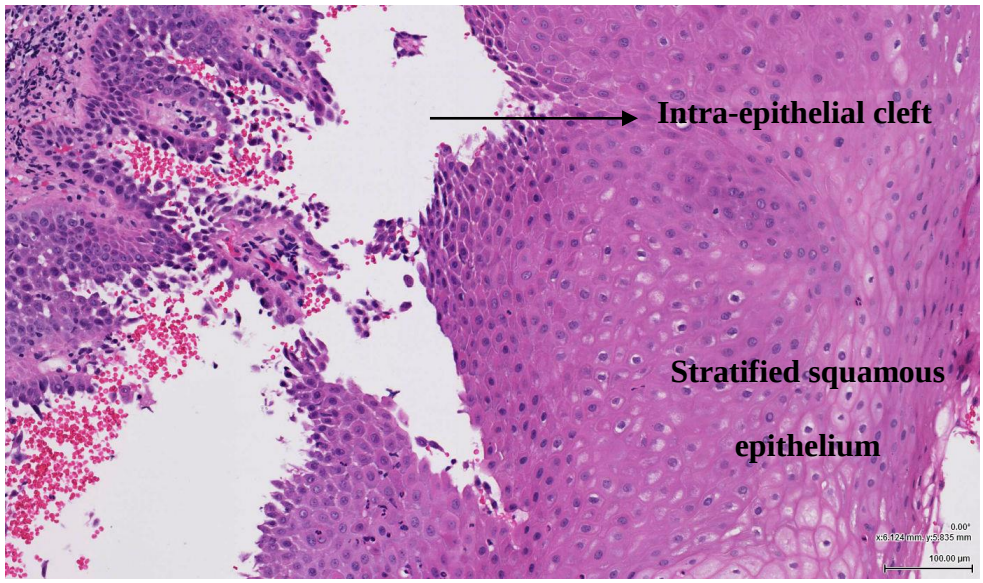


Figure 1-2 Histology of biopsy from an MMP and PV patient. a) Haematoxylin and eosin (H&E) stain of buccal mucosa in an MMP patient showing sub-epithelial separation (magnification 40.00x) b) Haematoxylin and eosin (H&E) stain of buccal tissue in a PV patient showing intra-epithelial separation (magnification 40.00x)

MMP is a predominantly mucosal disease affecting many possible sites. It is a heterogeneous disease with a wide variation in the number of affected sites as well as the disease severity. In sites such as the oral cavity, the consequences may be relatively mild while in others the consequences may be very severe. Scarring may result in blindness, oesophageal strictures or laryngeal stenosis. Furthermore there is wide

variation in the target antigens (Bagan J. et al., 2005). Therefore, early diagnosis is imperative to minimise the risk of complications associated with the disease.

Bullous diseases are a heterogenous group of disorders that predominantly affect the skin and/or mucous membranes. They are characterised by subepithelial blister formation and the target antigens lie within the basement membrane zone (BMZ). MMP is part of this spectrum of bullous diseases which also include bullous pemphigoid, epidermolysis bullosa acquisita and linear IgA dermatosis (Yeh et al., 2003).

Much work has been undertaken to establish the diagnostic criteria for MMP. The leading experts in the field put together the 1st international consensus in 1999 (Chan et al., 2002). Currently, direct immunofluorescence (DIF) is the gold-standard for diagnosis. Indirect immunofluorescence (IIF) may also be helpful for diagnosis and may be helpful in assessing the patient's response to treatment and disease activity. Enzyme-linked immunosorbent assay (ELISA) should allow determination of response to individual antigens, but is not routinely used on account of the presumed diversity of target basement membrane zone (BMZ) antigens. If defined clinical phenotypes could be related to specific antigens, this might provide a clearer understanding of the pathophysiology of the disease, and potentially, its prognosis and treatment.

The pemphigus group of diseases include pemphigus vulgaris (PV), pemphigus foliaceus (PF), IgA pemphigus and paraneoplastic pemphigus (PNP). Pemphigus foliaceus is a rare, skin disease with mucosal sites rarely involved. PV is the more common subtype of pemphigus. It shows less clinical and immunopathological diversity than MMP. The main target antigens are desmoglein 1 (Dsg1) for cutaneous lesions and desmoglein 3 (Dsg3) for mucosal lesions. It is clinically characterised by superficial blisters and chronic ulceration frequently affecting the oral mucosa. DIF is considered the gold-standard of diagnosis but IIF and ELISA with serum are routinely used to assess disease activity. While the mortality rate from PV has significantly decreased with the introduction of corticosteroids and adjuvant immunomodulating therapies, therapy is often associated with side effects such as infection and osteoporosis.

Oral lesions occur frequently in both MMP and PV and yet disease activity is not always associated with detectable circulating antibodies. An area of possible interest therefore, was to see whether disease specific autoantibodies may be detectable in saliva, and if so, to establish whether these might correlate with disease activity.

Studies to date have reported serum ELISA results in both MMP and PV (Kwon et al., 2008, Miyamoto et al., 2014). However, one study testing saliva in MMP showed no reactivity while a few have tested saliva in PV and have shown reactivity (Andreadis D, 2006, Hallaji et al., 2010, Hosseein Mortazavi, 2015).

The need for early and rapid diagnosis is important for a better disease outcome. More vital, the need for good prognostic biomarkers underpins the potential value in testing saliva as an alternative medium to serum. Furthermore, it provides an opportunity to better understand the disease pathogenesis and potentially the development of targeted treatment.

This project aims to establish whether saliva could provide a sensitive, reliable, cheap and non-invasive alternative to serum in the diagnosis and monitoring of MMP and PV. In addition, it aims to explore the relationship between specific phenotypes with autoantibody subclasses, antibody avidity and target antigens to more fully explain the spectrum of clinical presentation and disease severity.

1.1 Background

1.1.1 Historical Perspective

1.1.1.1 Mucous membrane pemphigoid (MMP)

In 1971, Hardy et al gave a concise review of the literature regarding MMP. It had been first described by Wichmann in 1794, in a case of eye involvement with chronic bullous disease. Then, in 1858, Cooper mentioned the first case with mild skin but severe scarring of the conjunctivae. Later, "Essential shrinkage of the conjunctivae" was reported in 1878 by Von Kries (Hardy et al., 1971). The term "chronic pemphigus of the mucous membranes" was introduced by Thost in 1896. Thereafter, in 1919, Thost recommended that "benign mucosal pemphigoid" be considered a separate entity (Shklar and McCarthy, 1959). In 1949, "Benign mucous membrane pemphigoid" was clearly established as a distinct form, after Civatte based this distinction on histopathological studies (Taylor, 1967). In 1951, Lever confirmed these findings finally separating benign mucous membrane pemphigoid from pemphigus vulgaris as distinct entities (Hardy et al., 1971). The term cicatricial pemphigoid was then introduced and appeared in the literature in the 1960's (Sneddon, 1962) and is still sometimes used. More recently, in 2002, the leading experts in the field designated the term MMP to a "group of putative autoimmune chronic inflammatory, sub-epithelial

blistering diseases predominantly affecting mucous membranes that is characterized by linear deposition of IgG, IgA, or C3 along the epithelial basement membrane" in the first international consensus for MMP (Chan L. et al., 2002).

1.1.1.2 Pemphigus vulgaris (PV)

It was first named by Wichman in 1791 when all blistering diseases were grouped together under one term, Pemphigus (Korman, 1988). Lever, in 1953, characterized PV, based on its histopathological picture, clinical criteria, and the course of the disease as a distinct entity from bullous pemphigoid (Korman, 1988). This was an important finding as it provided the base for separating the distinct clinical diseases, bullous pemphigoid and mucous membrane pemphigoid which form subepithelial clefts, from pemphigus which produces intraepithelial clefts. Not only that, it also demonstrated the difference between the pemphigus subtypes, as pemphigus vulgaris shows suprabasal clefts in contrast to pemphigus foliaceus where clefts occur in the sub-corneal layer (i.e. more superficial in the epithelium). The first hint to pemphigus's pathogenesis was identified in 1964 by Beutner and Jordon, who demonstrated that pemphigus patients' sera contained autoantibodies that bind to autoantigens present intercellularly in the skin and mucosa (Korman, 1988, Beutner et al., 1967).

1.1.2 Epidemiology

1.1.2.1 Mucous membrane pemphigoid

MMP has an estimated incidence of 1.3-2 per 1 million people per year in France and Germany respectively (Bernard et al., 1995, Bertram et al., 2009). It has a female predilection and a mean age of presentation between 60-65 years (Shklar and McCarthy, 1959, Hardy et al., 1971, Ahmed and Hombal, 1986, Silverman Jr et al., 1986, Schmidt and Zillikens, 2013). Very rarely, MMP has been known to affect children (Lara-Corrales and Pope, 2010, Kharfi et al., 2010, Cheng et al., 2001). Although geographical and racial predilections have not been highlighted in the literature, MMP seems to be more common in the European populations in comparison to others as clinically observed in this department.

1.1.2.2 Pemphigus vulgaris

There are several subtypes of pemphigus of which PV is the most common pemphigus subtype (Alpsoy et al., 2015). PV is a very uncommon disease with an incidence rate of

0.5-3.2/100,000 population/year (Korman, 1988). A recent study showed an 11% yearly average increase in the UK population (Langan et al., 2008). Geographical variations play a role in pemphigus incidence. Northern and Western Europe show a lower incidence of disease than Southern and Eastern Europe. Asian countries have a higher incidence than those in Europe (yearly incidence 1.6-16.1/million and 0.5-8/million respectively). The highest incidence was reported in Israel at 16.1/million (Alpsoy et al., 2015). This supports the previous association between pemphigus and Ashkenazi Jews and those of Mediterranean origins (Pisanti et al., 1974). It is extremely common in Northern India (Kanwar et al., 2006).

Pemphigus subtypes also differ in their geographical presentation. The endemic form, fogo salvagem, is common in Brazil, while 90% of the cases reported in India are PV (Wilson et al., 1993). With regards to age and sex distribution, PV has a wide range of onset and peaks at 50-60 years. Contrarily, it is only rarely reported in children and among these the mean age of presentation is 12 years (Lara-Corrales and Pope, 2010). Furthermore, PV is rarer still in neonates. Neonatal PV, which is caused by the passive transfer of autoantibodies (IgG) from the mother, is a transitory condition (Gushi et al., 2008, Fenniche et al., 2006). Sex predilection is also variable some reporting a female predilection while others report a male dominance mainly in middle-eastern countries (Alpsoy et al., 2015).

1.2 Clinical Presentation

1.2.1 Mucous membrane pemphigoid

MMP has a wide spectrum of clinical presentation. It includes patients with oral lesions alone, ocular involvement alone, and those with multiple mucosal sites, with or without skin involvement. Patients with pure oral disease; which are further sub-categorised to gingival (24%), extra-gingival (12%), or combined (51%) are considered to have a more mild form in comparison to those with ocular involvement who may develop severe consequences (Setterfield, 2009). The majority of patients however, generally present with multi-site involvement as demonstrated in (Fig 1-3 and 1-4). The disorder follows a chronic course with episodes of acute exacerbation and remission.

The overall distribution of site involvement varies between studies. However in a review of 16 case series (457 cases) the sites of involvement included oral mucosa 85%, conjunctiva 64%, skin 24%, nasal mucosa 15%, pharynx 19%, anogenital 17%, larynx 8%, and oesophagus at 4% (Ahmed and Hombal, 1986).

a)

b)

c)

Figure 1-3 Clinical presentation of MMP a) MMP patient with desquamative gingivitis. b) MMP patient with inflammation and blister on the palate. c) MMP patient with skin involvement which is often associated with scarring seen here resulting in alopecia

a)

b)

Figure 1-4 Ocular MMP demonstrating a) subconjuctival inflammation and shrinkage of the tarsal conjunctiva b) symblepharon formation

1.2.2 Pemphigus vulgaris

PV is a mucocutaneous disease resulting in superficial blisters and chronic ulceration. Mucosal surfaces involved may include the oral cavity, conjunctiva, genitalia, and upper respiratory tract (Hasan et al., 2011). Classically, lesions appear to be flaccid, thin-walled bullae that may form on normal or erythematous skin and/or mucous membranes. They break leaving partially denuded areas of variable size which enlarge as the epithelium detaches from the periphery. Commonly, skin lesions are seen as erosions and heal without scarring. Skin areas affected are usually the trunk, pressure areas, groin, and axillae (Razzaque, 1980). The first sign of PV in 60% of the cases is oral lesions. At some point over the course of their disease, 80-90% of the patients will develop oral lesions as reviewed by (Greenberg and Glick, 2003, Hasan et al., 2011). The most predominant sites of involvement are the gingivae, buccal and palatal mucosa as shown in (Fig 1-5).

a)

b)

c)

Figure 1-5 Clinical features of pemphigus vulgaris a) Oral lesions in a PV patient with extensive ulceration of the hard palate b) Buccal mucosal ulceration with surrounding mucosal fragility c) Skin involvement in a PVpatient showing ulceration with crusted edges

1.3 Aetiopathogenesis

1.3.1 Mucous membrane pemphigoid

The precise pathogenesis of MMP is unclear. Much of the work regarding pathogenesis of subepidermal blistering diseases has been undertaken on a closely related disorder bullous pemphigoid (BP). Recently two active mouse models have been developed which reflect the roles of auto-reactive T cells in the pathogenesis of BP (Ujiie et al., 2010, Hayashi et al., 2011). They demonstrated that patients with the *HLA-DQB1*03:01* (formerly known as *HLA-DQB1*0301)* allele produced both T helper (Th) 1 and Th2 cytokines (e.g. IL-2 and IL4 respectively). Furthermore, the cytokines IL-6 and IL-8 as well as tumour necrosis factor (TNF), have been detected in serum samples of BP patients and have been reported to reflect disease activity (Schmidt and Zillikens, 2013). The pathogenic role of anti-BP180 antibodies (see section 1.5) was established in a bullous pemphigoid animal model when sub-epidermal blistering was induced by passive transfer (Liu et al., 1993). The subsequent finding that the BP antigens are also targeted in MMP by implication has suggested that animal model studies may in part help with the understanding of the pathogenesis of MMP (Liu et al., 1993).

MMP is an autoantibody complement mediated disease which results in cytokine and leukocyte enzyme release thus detaching the basal cells from the BMZ (Bagan J. et al., 2005). Autoantibodies are produced after the presentation of self-antigens which lead to T helper cell activation. This in turn is followed by B cell activation. Subsequently, autoantibodies are produced against the presented antigen by plasma cells. IgG and/or IgA are usually detected with IgG4 as the most common subtype (Black et al., 2004, Hertl et al., 2006). Combined circulating IgG and IgA anti-BMZ autoantibodies have been shown to correlate with disease severity (Setterfield J. et al., 1998, Rabelo et al., 2014). Furthermore, sequential titres of circulating IgG have been associated with disease activity (Setterfield et al., 1999). These results were established by IIF titres. Studies including a large number of MMP patients and testing for different antigen reactivity have not been well established.

An immunogenetic association with *HLA-DQB1*03:01* is reported in the literature (Setterfield et al., 2001, Chan et al., 1997, Mondino et al., 1978). This may contribute to the T-cell recognition of basement membrane antigens. Changes in major histocompatablity complex (MHC) which were experimentally induced in animal models, showed an effect on the development of autoimmune diseases (Theofilopoulos,

1995). However, the precise role in the pathogenesis has not been established as there are patients with the disease but without the allele (Setterfield et al., 2001).

Although the mechanism is unclear, drugs such as furosemide have been reported to trigger the disease (Van Joost and Van't Veen, 1993). In addition, penicillamine has been shown to produce adverse reactions of altered immunity resulting in penicillamine-induced cicatricial pemphigoid (Bialy-Golan and Brenner, 1996).

Cytokines may play an important role in the scarring process of the disease. Interleukins (IL) IL-3, IL-4, IL-5 and Tumour growth factor beta (TGFβ) contribute to the scarring which occurs in MMP (Caproni et al., 2002, Caproni et al., 2003, Carrozzo et al., 2014). Furthermore, IL-6 and IL-8 release is triggered by anti-BP180 IgG antibodies which are linked to Th 2 cells (Schmidt et al., 2001a). In addition, regulatory T cells (Treg) have been proposed to contribute to the chronicity of MMP as they were expressed in MMP lesions (Torchia et al., 2009).

1.3.2 Pemphigus vulgaris

An association between PV and MHC class II genes has been reported in the literature; *HLA-DR4*, *DQ8*, *DR6* and *DQ5* have been implicated (Bhol et al., 1994). Antigen mimicry may be responsible for the appearance of conditions that resemble PV. This has been shown to occur in bacterial and viral infections such as Staphylococcal infections or Varicella (Amagai, 2003). In addition, pemphigus-like lesions may result from drugs whether they contain sulfhydryl groups as captopril or not (Tchernev and Orfanos, 2006).

Autoantibodies have been documented as pathogenic in various studies (Anhalt et al., 1982, Amagai et al., 2000). The fact that antibodies correlate with disease severity by IIF suggests that these intercellular antibodies are pathogenic. In addition, since neonatal autoantibodies cause transient neonatal pemphigus, this further suggests that passively transferred antibodies from the mother are pathogenic. Moreover, pathogenicity is supported by plasma exchange studies where there is clinical improvement associated with a decrease in antibody titre, (Ruocco et al., 1978) and normal human skin explants incubated with PV serum *in vitro*, resulting in the appearance of suprabasal acantholysis (Michel and Ko, 1977). The development of an *in vivo* model where the IgG fraction of serum from PV patients was injected into neonatal mice resulted in the clinical features of PV provided further evidence of pathogenicity (Anhalt et al., 1982).

Since Dsg3 is the main isoform expressed in mucous membranes, anti-Dsg3 antibodies alone are sufficient to cause mucosal lesions. However, both Dsg 1 and 3 are expressed in the skin and therefore both antibodies are needed to result in mucocutaneous lesions of PV (compensation theory) (Amagai et al., 2006). Therefore, anti-Dsg3 antibodies are detected in patients suffering from mucosal lesions, while anti-Dsg1 and 3 are detected in those with both skin and mucosal involvement. In addition, the levels of anti-Dsg antibodies in serum reflect disease activity (Amagai et al., 1999, Cheng et al., 2002).

Whether these autoantibodies alone are responsible for the disease pathogenesis is still controversial. Since Th2 cells direct autoantibody production, they are associated with disease activity (Amagai et al., 2000). The IgG4 subtype has been detected in active pemphigus which may support the Th2 involvement in the pathogenesis of the disease (Bhol et al., 1994, Wilson et al., 1993). Tregs play a suppressive role in antigen-specific antibody production (Yokoyama et al., 2011). Whether antibodies are behind the pathogenesis of the disease or whether they are 'witnesses of the disease', still remains a point of debate in the literature as does the process of acantholysis (Amagai et al., 2006).

IgA pemphigus is a rare subtype of pemphigus. It is characterised by detection of IgA antibody instead of IgG and is usually directed against desmocollin (Dsc) and in a few cases to the Dsg1 and/or 3 antigen (Tajima et al., 2010, Hashimoto, 2003). Although uncommon, there have been some reports in which IgG driven PV has also shown IgA antibodies to the PV antigens (Kowalewski et al., 2006). There is much debate about IgA pemphigus in terms of nomenclature and its' characteristics (Hashimoto and Nishikawa, 2015). Furthermore, IgA autoantibody production and their role in pathogenicity of the disease is still unclear (Hashimoto, 2001).

1.4 Autoantibody Detection

1.4.1 Immunofluorescence

1.4.1.1 Direct immunofluorescence (DIF)

Direct immunofluorescence is a technique which entails pairing of the patient's biopsy specimen (mucosal or skin) with a fluorophore labelled immunoglobulin (primary antibody). Subsequently, the specimen is then studied under a fluorescent microscope. The results depend on the wavelength emitted once excitation occurs. This technique depends on the specificity of autoantibodies to certain target antigens thus enabling the

visualization of the distribution of these specific antibodies to the target antigen within the specimen (Sciubba, 2011).

1.4.1.1.1 Mucous membrane pemphigoid

Although DIF is technique sensitive, it is the gold standard for the diagnosis of MMP as most studies show that 80-97% of specimens are positive (Fine et al., 1984, Ahmed et al., 1991). However, it is important to note that pure ocular disease patients have a lower positivity with over 40% having a negative DIF (Thorne et al., 2004, Bernauer et al., 1994, Kirzhner and Jakobiec, 2011). Therefore, in cases with multisite involvement a biopsy of another mucosal lesion is more likely to produce a positive result. DIF studies of peri-lesional skin or mucosa demonstrate a linear, continuous band at the basement membrane zone (BMZ), more often with IgG (90%), C3 (90%), IgA (80%), and IgM (70%) (Bean et al., 1972, Griffith et al., 1974, Rogers et al., 1977, Fine et al., 1984, Meyer et al., 1985) as demonstrated in (Fig 1-6).

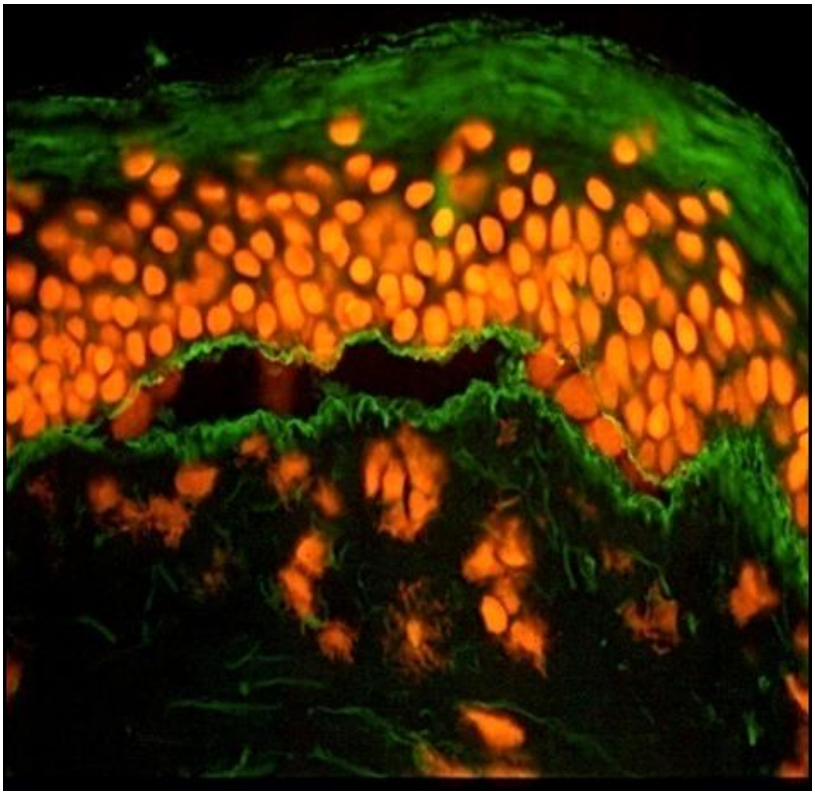


Figure 1-6 DIF from an MMP patient (buccal mucosal sample) showing a linear band of IgG, C3 and /or IgA along the basement membrane zone (BMZ) (roof or floor binding depicts the heterogeneity of targeted antigens)

1.4.1.1.2 Pemphigus vulgaris

The gold standard of diagnosis is DIF which provides greater sensitivity than IIF. Any areas biopsied will generally result in a positive DIF. Patients who are in remission

may also still show a positive DIF (Judd and Lever, 1979). IgG and C3 bind to the keratinocyte cell surface throughout the epidermis (Sami, 2011, Jordan Re, 1971). Inter-cellular binding of IgG and/ or C3 is found in the epithelium (Schmidt and Zillikens, 2010, Korman, 1988) as demonstrated in (Fig 1-7).

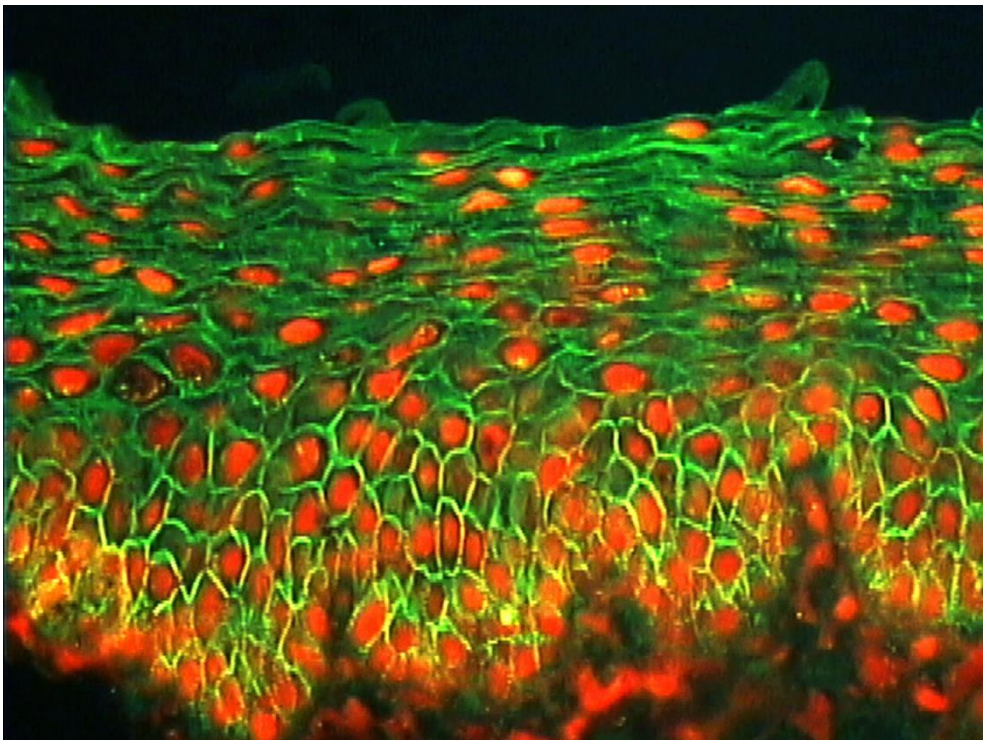


Figure 1-7 DIF from a PV patient (buccal mucosal sample) showing intercellular deposition of IgG and/or C3 in a characteristic 'chicken wire' pattern

1.4.1.2 Indirect immunofluorescence (IIF)

In order to quantify the autoantibody titre present in the patient's serum, indirect immunofluorescence is used. The technique involves serially diluting the patient's serum which is then reacted with a tissue substrate using a labelled anti-human immunoglobulin. Unlike the DIF technique, which only utilises a primary conjugated antibody, IIF reaction depends on using both a primary and a secondary antibody. First a non-conjugated primary antibody (serum sample) is applied which binds specifically to the target antigen. Subsequently, a secondary antibody linked to a fluorophore, binds to the primary antibody after recognising it (Sciubba, 2011).

1.4.1.2.1 Mucous membrane pemphigoid

Circulating IgG autoantibodies were first reported by Dantzig with a titre of 1:40 (Dantzig, 1973). Early IIF studies used animal tissues including monkey oesophagus or

guinea pig lip and these infrequently showed circulating antibodies (0-36%) (Bean et al., 1972, Griffith et al., 1974). By using salt-split skin (SSS) (0.1 mol/L salt split skin or mucosa) the sensitivity of IIF improved to 88-100% (Gammon et al., 1984, Kelly and Wojnarowska, 1988). The binding patterns of circulating autoantibodies in patients with MMP may occur on the epithelial side of the split, dermal, or combined reflecting the variation in autoantigens detection by autoantibodies (Chan et al., 2002). IgG4 and IgG1 are the predominant subclasses in MMP (Bernard et al., 1991). Some studies report 20-30% circulating autoantibodies (Leonard et al., 1984, Ahmed et al., 1991, Leonard et al., 1988), while others have up to 90% positivity for both IgG and IgA (Setterfield J. et al., 1998, Setterfield et al., 1999, Murakami H. et al., 1998).

1.4.1.2.2 Pemphigus vulgaris

Unlike MMP, guinea pig or monkey oesophagus substrates are successful in PV and are used for IIF testing in PV (Harman et al., 2000b, Yeh et al., 2003). Circulating IgG autoantibodies to Dsg3 and 1 show a characteristic "chicken wire" pattern predominantly on the lower most epithelial cell layers (Fig 1.7). It is considered to be a less sensitive technique for PV than DIF but provides a prognostic value as titres of circulating antibodies correlate with disease activity (challacombe S. J. et al., 2001).

IgG is detected in 80-90% of the patients and correlates with disease activity (Sami, 2011). Tissue bound and circulating antibodies mainly fall into the IgG1 (T helper 1 regulated) and IgG4 (T helper 2 regulated) subclasses (Hertl et al., 2006). It has also been reported, that both IgG4 and IgG1 are present in high titres in the acute phase of the disease, while IgG1 is found in remission and healthy individuals (Bhol et al., 1994).

1.4.2 Enzyme-linked immunosorbent assay (ELISA)

Engvall and Perlmann in 1972 reported a sensitive and simple method for the quantitative determination of autoantibodies (Engvall and Perlmann, 1972). The indirect ELISA technique involves the binding of an antigen by incubation to a micro-titre plate. This antigen solid phase is used to bind specific antibodies of a test sample. Unbound material is removed by washing and antibody is detected using an enzyme labelled anti-immunoglobulin. This is the type of ELISA used in detecting autoantibodies targeted against specific antigens in MMP and PV. ELISA's are now arguably more sensitive than IIF as they are less technique sensitive (Ishii et al., 1997).

1.4.2.1 Mucous membrane pemphigoid

Since the introduction of ELISA in the early 1990's, this technique has been used frequently to aid in the diagnosis of immunobullous diseases. ELISA is now being more commonly utilized to detect BMZ components, which are identified by the patients' sera (Hayakawa et al., 2014). Pre-coated commercial ELISA plates are now available using recombinant BP180 (Calabresi et al., 2007, Sciubba, 2011). There is much debate about the pathogenesis of MMP in relation to autoantibodies to BP180 in MMP. However it has been shown to be the main target antigen in a large study of MMP patients (Oyama et al., 2006).

The use of ELISA for serum studies in MMP is well described. However, only one study thus far has used saliva as a fluid for autoantibody detection in MMP (Andreadis D, 2006). The authors reported that saliva testing for MMP was unhelpful. They argued that their use of pre-coated plates with the recombinant NC16a domain of BP180 was the reason for the negative results (Andreadis D, 2006). However numerous studies have described IgG reactivity with this epitope of BP180 in serum (Balding et al., 1996, Calabresi et al., 2007, Chan et al., 2002, Csorba et al., 2011).

Research into the pathogenesis of BP has paved the way for a better understanding of the pathogenesis of MMP. Notably a recent study has been published testing for salivary antibodies to BP180-NC16a in BP patients. Esmaili et al reported that in 50 new patients tested for reactivity to both BP180-NC16a and BP230 ELISAs using both serum and saliva were both specific and sensitive for diagnosis. In addition, salivary anti-BP180-NC16a antibodies were associated with disease severity. They also reported that serum and salivary IgG anti-BP230 antibodies were associated with mucosal severity (Esmaili et al., 2015). This is surprising as mucosal involvement in BP is usually mild and transient. This raises the possibility that these patients may have been MMP with skin lesions. Further studies are therefore needed.

1.4.2.2 Pemphigus vulgaris

ELISA is a well-established method of measuring serum autoantibody titres to Dsg3 and Dsg1, which directly correlate with mucosal involvement and skin involvement respectively in PV (Harman et al., 2000a). ELISA is also used to monitor sequential serum antibody levels over the course of the disease (Schmidt and Zillikens, 2010).

There are only three published studies using saliva. Andreadis et al found that salivary anti-Dsg1 and 3 IgG ELISA are both sensitive and specific, thus suitable for the diagnosis of pemphigus (Andreadis D, 2006). In addition, Hallaji et al similarly reported salivary Dsg1 and 3 ELISA could be used for the diagnosis of PV (Hallaji et al., 2010). More recently, Mortazavi et al published a 90% specificity and 70% sensitivity of saliva anti-Dsg antibody detection (Hosseein Mortazavi, 2015). However, further studies are now indicated to study paired salivary and serum titres and their potential use in disease monitoring.

1.5 Target Antigens

The basement membrane links epithelial cells to the underlying connective tissue and is fundamental to maintaining the structural integrity of the skin or mucous membranes. Target antigens to which autoantibodies are produced, lie within the hemidesmosomal region of the basement membrane zone in MMP. In PV the target antigens, are found within the epithelial layer in a region called the desmosome as shown in (Fig 1-8).

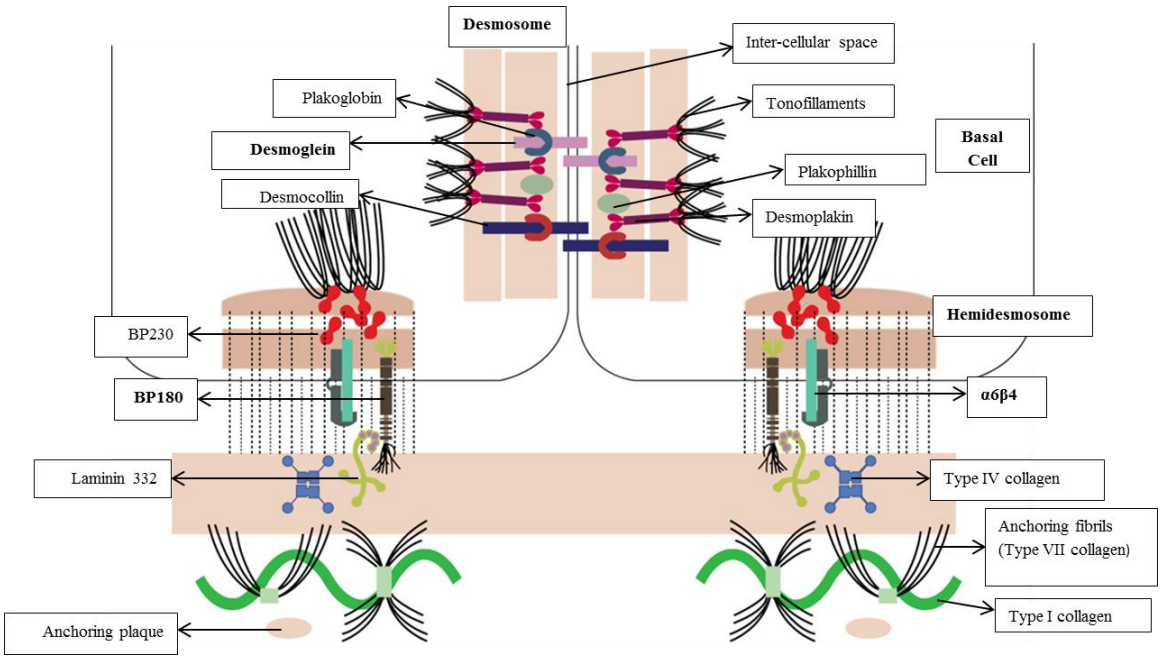


Figure 1-8 Structure of desmosome and hemidesmosome. The figure displays the basal cell and underlying connective tissue. The desmosome and hemidesmosome are home to antigens targeted in both PV and MMP, respectively. Modified from (Taghipour K. and Wojnarowska, 2009)

1.5.1 Mucous membrane pemphgoid

Hemidesmosomes are specialized junctional complexes that provide attachment between epithelial cells and the underlying basement membrane. They maintain cell polarization, organization and tissue architecture (Borradori and Sonnenberg, 1999). They are small electron dense domains (< 0.5 μm) located at the surface of basal keratinocytes. Hemidesmosomal plaque, anchoring filaments and anchoring fibrils form the functional unit known as the hemidesmosomal adhesion complex. This provides a stable attachment of keratinocytes to the underlying basement membrane (Zillikens, 1999). Any interference that occurs to this complex results in disruption of the tissue architecture and causes split formation at the level of the dermal-epidermal junction (Zillikens, 1999, Borradori and Sonnenberg, 1999).

A number of hemidesmosomal antigens have been implicated in the pathogenesis of MMP. These include BP180 (75%), BP230 (25%), laminin 332 (25%), α6β4 integrin, and type VII collagen (Schmidt and Zillikens, 2013). The major target antigen is BP180. While specific clinical subtypes have been associated with specific target antigens, e.g. ocular MMP and the β4 integrin (Tyagi et al., 1996) and oral MMP with the α6 integrin (Chan, 2012), others have found conflicting results. Some multi-site disease patients have been reported to show IgG antibody reactivity to the β4 integrin (Leverkus et al., 2001). In addition, in the pure ocular sub-group, antibody reactivity was reported with both β4 and the C-terminal domain of BP180 (Solano-López et al., 2014). However, in cases of oral MMP, the target antigen is still unclear; Rashid et al in 2006 studied 30 patients whom 15 had oral involvement alone and showed that all reacted with the α6 integrin (Rashid et al., 2006a). Moreover, Bhol et al found that 16 of 20 cases of oral pemphigoid reacted with the α6 integrin (Bhol et al., 2001). Contrary to those findings, Calabresi et al studied 20 cases limited to the oral cavity in whom 75% of the cases reacted with BP180 (Calabresi et al., 2007). Carrozzo et al in the 28 cases studied showed 46% reacting with BP180 (Carrozzo et al., 2004). Schmidt et al in 2001 reported that autoantibodies target different epitopes of BP180 in the 26 cases studied (Schmidt et al., 2001b). This shows the controversy surrounding target antigens and specific phenotypes. Further research is needed in this area.

Since the majority of cases target BP180, this will be the main antigen to be studied in this project together with the α6β4 integrin. As the α6 and β4 fragments' associations may be linked to specific clinical phenotypes. These two antigens are described in

further detail below. BP230 and laminin 332 are also considered major antigens of MMP and are described below. Collagen VII on the other hand, has not been described because of the overlap with epidermolysis bullosa acquisita (EBA) as it is considered the major antigen for that disease. The table below shows the heterogeneity of MMP and the different antigens targeted (Table 1-1).

Table 1-1 Summary of main Clinical and Immunological features of mucous membrane pemphigoid

Study by	Study No.	Site of involvement	Antibody isotype	Antigen	Percentage positive	Test used
(Tyagi et al., 1996)	n=12	ocular	IgG	β4	12/12 100%	Immunoblot
(Murakami H. et al., 1998)	n=50	Oral and ocular	IgG	BP180-NC16a	18/50 36%	Immunoblot
				C-terminal domain (BP180-4575)	25/50 30%	
				BP230	7/50 14%	
			IgA	C-terminal domain (BP180-4575)	1/50 2%	
(Bhol et al., 2001)	n=20	Oral	IgG	α6	16/20 80%	Immunoblot
(Kumari et al., 2001)	n=20	Ocular	IgG	β4	20/20 100%	Immunoblot
(Schmidt et al., 2001b)	n=26	Multisite	IgG	Laminin 332	5/26 19%	Immunoblot
	n=19			BP180-NC16a	10/19 53%	
				BP180-C terminal (4575)	3/19 16%	
			IgA	BP180-NC16a	4/19 21%	
				BP180-C terminal (4575)	6/19 32%	
(Egan et al., 2003)	n=35	Multisite	IgG	Laminin 332	24/35 69%	Immunoblot
(Carrozzo et	n=28	Oral(+/-	IgG	BP180	13/28 46%	Immunoblot

al., 2004)		mucosal)				
				BP230	6/28 21%	
			IgA	BP180	3/28 11%	
				BP230	3/28 11%	
(Sakuma-Oyama et al., 2004)	n=124	Multisite	IgG	BP180 full length	75/124 27%	Immunoblot
				BP230	34/124 27%	
				β4	26/124 21%	
(Rashid et al., 2006a)	n=30 (n=15) (n=15)	Ocular(+/- mucosal) Oral	IgG	α6	15/15 100%	Immunoblot
				β4	15/15 100%	
(Rashid et al., 2006b)	n=20	Oral	IgG	α6	20/20 100%	Immunoblot
(Calabresi et al., 2007)	n=20	Oral	IgG	BP180	6/20 30%	Immunoblot
				BP180 C-terminal domain	4/20 20%	
				BP230	5/20 25%	
				200 Kda (β4)	3/20 15%	
			IgA	BP180	1/20 5%	
				BP230	2/20 10%	
			IgG	BP180-NC16a	9/20 45%	ELISA
				BP180-mid portion	3/20 15%	
				BP180-C terminal domain (4575)	4/20 20%	
(Groth et al., 2011)	n=35	Mucosal and skin	IgG	Laminin 332	24/35 69%	ELISA

A-Bullous Pemphigoid antigen 2 (Collagen XVII) (BP180)

BP180 is a transmembrane glycoprotein with a type II orientation; the carboxyl (COOH) terminal portion is extracellular (1007 amino acid (aa)) shown in (Fig 1-9). The amino (-NH2) terminal is the intracellular segment (466 aa). The extracellular portion consists of 15 collagenous domains of various sizes which are separated by short non-collagenous sequences of 500 residues.

There are two main epitopes identified in the literature; the NC16a domain and 4575-segment (C-terminal domain). NC16a is the first non-collagenous segment in the extracellular part of BP180. It is a 72 amino acid stretch (490-562) and it contains the NC16a domain (490-534). The 4575 epitope, is a 48 aa segment further down BP180, adjacent to the carboxyl terminal (1365-1413) (Zone et al., 1998, Powell et al., 2005). The extracellular ligand of BP180 attaches to the α6 integrin, while the intracellular portion is associated with the β4 integrin (Powell et al., 2005). The majority of patients show circulating IgG antibodies specific to BP180. Some sera contain IgA antibodies which are reactive with a 97/120 Kda protein, the linear IgA bullous dermatosis (LABD) antigen 1 which is homologous to the extracellular portion of BP180 confirming heterogeneity of MMP (Christophoridis et al., 2000, Parisi et al., 2003, Zone et al., 1998, Murakami H. et al., 1998).

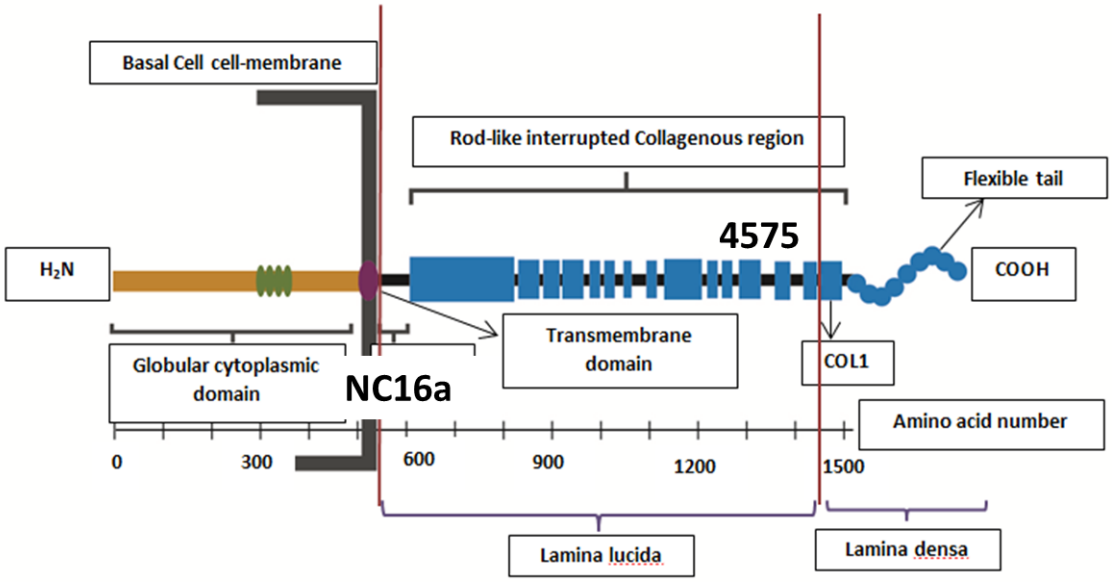


Figure 1-9 Structure of BP180 The figure depicts the intracellular domain, transmembrane domain and the extracellular region of BP180 highlighting the epitopes which are considered the most antigenic (NC16a and 4575)**.** Modified from (Zone et al., 1998)

B-Alpha 6 Beta 4 Integrin (α6β4)

The α6β4 integrin is a cell surface glycoprotein complex. As a member of the integrin superfamily, it mediates cell-cell and cell-matrix interactions. Integrins are heterodimers of non-covalently linked alpha and beta transmembrane glycoproteins (Tamura et al., 1990). Most strongly expressed in stratified squamous epithelium, alpha 6 is present in the entire surface of basal cells although strongly localized to the basal

region. Beta 4 is found mainly in the basal region. As this integrin is localized to the hemidesmosome, it provides an important role in binding epidermal cells to the basement membrane (Sonnenberg et al., 1991). Furthermore, it shows specific roles in cell signal transduction pathways in both the central and peripheral nervous systems, it is associated with cytoskeletal and signalling molecules and is considered a potential target for cancer molecule therapy (Su et al., 2008).

The α6 subunit contains 1073 aa residues and is very similar to various alpha subunits cloned. The β4 subunit however, has a unique cytoplasmic domain, which consists of 1778 aa (683 aa extracellular domain, 23 aa transmembrane domain and 1072 intracellular cytoplasmic domain) (Tamura et al., 1990). Specific interactions of the β4 subunit and cytoplasmic proteins have been documented (Suzuki and Naitoh, 1990). The extracellular domain of α6β4 binds laminin 332 while the carboxy terminus binds BP180 (Giancotti, 1996).

C- Bullous Pemphigoid antigen 1 (BP230)

The plakin family of proteins are involved in the organisation of the cytoskeletal architecture and include BP230 and plectin (Borradori and Sonnenberg, 1999). BP230 was first recognized as a target antigen for bullous pemphigoid (BP) in 1981 (Stanley et al., 1988). It is an intracellular component of the hemidesdmosomal plaque (Desai et al., 2008). The COOH terminal is associated with intermediate filaments indicating a role of BP230 in the attachment between the intermediate filaments and hemidesmosomal plaque. The NH_2 terminal of BP230 has been reported to interact with the cytoplasmic domain of BP180 (Borradori and Sonnenberg, 1999). It is targeted in MMP in about 25% of the cases and usually with BP180 reactivity (Schmidt and Zillikens, 2013). BP230 pre-coated ELISA kits are available for testing sera (Lee et al., 2012).

D- Laminin 332

Laminin 332 is a heterodimeric glycoprotein which consists of 3 subunits α3 (200-165 Kda), β3 (140 Kda) and γ2 (155-105 Kda). These subunits are covalently linked with disulphide bonds. It plays an important role in the attachment of basal cells to the underlying connective tissue (Bekou et al., 2005). It is estimated at 460 Kda and has previously been known as nicein, kalinin, epiligrin and laminin 5 (Domloge-Hultsch et al., 1994). It is produced by epithelial and mesenchymal cells and is found in the skin,

trachea, cornea and oesophagus for example. Gene defects are known to cause Herlitz and non-Herlitz junctional epidermolysis bullosa acquisita (Vailly et al., 1995).

Passive transfer of the IgG fraction containing anti-laminin 332 antibodies from sera of MMP patients has been shown to cause subepidermal separation and blister formation (Lazarova et al., 1996). All three subunits have been shown to react with MMP sera however the most common reactivity is with the α3 subunit (Bekou et al., 2005, Schmidt and Zillikens, 2013). ELISA tests should be conducted for dermal binding sera in light of the association between an underlying malignancy and anti-laminin 332 MMP (Bekou et al., 2005, Bernard et al., 2013).

1.5.2 Pemphigus vulgaris

Desmosomes are multiprotein complexes. They form the intercellular attachment structures that are linked to the intercellular intermediate filament network. They are expressed mainly by epithelial cells and are most commonly present in tissue undergoing mechanical stresses such as the skin, heart and bladder (Payne et al., 2004). They maintain cell cohesion. They are disc-shaped organelles of 0.1-0.5 μm diameter. Heterophilic interactions between transmembrane glycoprotein desmocollins and desmogleins (Dsc and Dsg) are thought to form the basis of cell-cell adhesion (Schmidt A. and Koch, 2007).

Cadherins (calcium dependent cell adhesion molecules) are transmembrane components of the desmosome. They are responsible for intercellular adhesion and interact with intermediate filaments. There are two main groups identified; Dsg and Dsc which contain an extracellular portion. They bind to the desmosomal plaque through the intracellular fragments. Anti-desmoglein pathogenic antibodies are usually the culprit of cell-tissue attachment disturbances which clinically result in mucocutaneous lesions (Tchernev and Orfanos, 2006).

Dsg3 has been identified as the PV antigen (Amagai et al., 1991, Eyre and Stanley, 1988). Dsg3, 130 KDa, is a transmembrane protein belonging to the cadherin family. It comprises of five extracellular domains, a transmembrane domain, and an intracellular portion. The major antigenic regions of Dsg3 are EC1, EC2, and EC4 (Gniadecki, 2006). Dsg3 and Dsc1 are implicated in the IgA subtype of pemphigus. Other antigens may be targeted such as cholinergic keratinocyte receptor, desmoplakin, endoplakin, periplakin and plectin (Tchernev and Orfanos, 2006).

Dsg1 is a 160 KDa transmembrane molecule and is part of the cadherin family. The mature form consists of five extracellular domains EC1-EC4 and an extracellular anchor domain attached to the transmembrane region. The intracellular domain is made up of five domains. It is abundantly found in stratified epithelium of the epidermis, tongue and oesophagus. Dsg1 is considered the PF antigen (Gniadecki, 2006, Eyre and Stanley, 1988).

1.6 Mucosal immunity

Mucosal immunity represents the portion of the immune system which provides protection within the mucous membranes. It protects the mucosa from infection, prevents the uptake of microbes and moderates the immune response. The discovery of a distinct immunoglobulin (IgA) and later the epithelial glycoprotein, which is known today as secretory component (SC) that acts as a membrane receptor, were the starting points of understanding mucosal immunology. A difference was constantly shown between humoral response at mucosal surfaces and that of serum. Therefore the notion that a local mucosal immune system which is structurally and functionally separate from the systemic counterpart was evidenced by the characteristic mucosal immune response at mucosal surfaces, and by the distinct immunoglobulin isotype serving as the major mucosal antibody (Tomasi, 1972).

The mucosal immune system is commonly described as mucosa-associated lymphoid tissue (MALT). It is also classified according to the type of mucosa, i.e. gut-associated lymphoid tissue (GALT) and nasal-associated lymphoid tissue (NALT). Mucosal surfaces in the body include the respiratory mucosa, the gut, oral and ocular mucosa as well as the vagina (Janeway CA Jr, 2001).

One of the main characteristics of the mucosal immune system is that IgA induced at one mucosal site can be detected at another mucosal site with IgA secreting B cells. The term “common mucosal immune system” is therefore an apt description. This is also supported by the fact that the mucosal system is divided into inductive and effector sites. Common induction sites include tonsils, adenoids and Peyer’s patches (Mantis et al., 2011). Effector sites are represented by the lymphocytes and plasma cells scattered within the lamina propria at the mucosal sites (Janeway CA Jr, 2001). It is noteworthy to mention that some studies have reported germinal centre reactions in non-lymphoid target tissue such as salivary glands in Sjögren Syndrome (Stott et al., 1998, Szodoray et al., 2005). Secretory IgA (SIgA) is the most dominant immunoglobulin in the mucosal

immune system. In serum, monomeric IgA is most commonly found while dimeric IgA is most abundant in mucosa (Mestecky, 1987). Monomeric IgA is 150 Kda. SIgA contains the J chain (15 Kda) which links two IgA molecules in addition to the SC (70 Kda) resulting in a molecular weight of 385 Kda.

The role of mucosal immunology has been extensively studied especially with regards to the gut and vaccines (MacPherson et al., 2008, Holmgren and Czerkinsky, 2005). Since MMP is mainly a mucosal disease where mucosal surfaces are involved; the presence of SIgA may be very relevant. Mucosal antibodies have not been studied before in MMP. Oral MMP, although a milder form of the disease, tends to take a long time to heal. Thus the potential presence of mucosal antibodies may have particular relevance to the pathophysiology of the disease. Similarly in PV, the oral mucosa is one of the main sites affected and therefore the presence of mucosal antibodies would also be of significant interest.

1.7 Saliva

1.7.1 Composition

Saliva in humans is a dynamic fluid. It possesses several important functions for maintaining oral health and homeostasis. Oral fluid originates from three major salivary glands (parotid 23%, sublingual 4%, and submandibular 65%) as well as from a large number of minor salivary glands (8%) (Chiappin et al., 2007).

In addition, saliva contains a non-glandular component. It encompasses fluids originating from oro-pharyngeal mucosa, gingival crevicular fluid (GCF) derived from the epithelium of the gingival crevice and produced at 2-3 µl/h per tooth, food debris, and blood-derived compounds. The normal adult produces 500-1500 ml of saliva per day, approximately 0.5 ml/min. This can be affected by physiological and pathological conditions. Saliva consists mainly of 98% water and 2% comprising mucous, enzymes, electrolytes, proteins, and antibacterial components.

The salivary immunoglubulins are mainly SIgA which make up >85% of the total immunoglobulin in saliva. They are produced directly by B lymphocytes located near salivary glands and 35% of SIgA is produced by minor salivary glands. The remaining 5-15% of immunoglobulin comprise of mainly IgG and IgM which are, to some extent, derived from gingival crevicular fluid (GCF) or from plasma leakage (Van Nieuw Amerongen et al., 2004). However, >70% of IgG in saliva is thought to be of local origin; produced by palatine, parotid, lingual, adenoids, and tonsillar plasma cells

(Butler et al., 1990). It has been estimated that crevicular fluid washings (CFW) contributes 1-2 ml/day into saliva and that the IgG concentration approaches that of serum (Challacombe et al., 1978). In terms of monomeric IgA 77% is derived from serum (Mestecky, 1987).

Salivary components are not only important for oral cavity functions; they also provide clues to systemic conditions. Importantly salivary IgA was shown to be stable under storage conditions (Ng et al., 2003).

1.7.2 Potential use of biomarkers in saliva

The term biomarker is used to refer to a specific substance or molecule which is considered a measurable indicator to a certain condition. Because serum components of saliva are derived primarily from the local vasculature; saliva offers a fluid source that provides many, if not most, of the same molecules found in the systemic circulation, thus having potential in clinical and research applications. It has been used to test for antibodies to bacteria and viruses, hormones (oestrogen, testosterone), environmental toxins, alcohol, tobacco, and certain drugs (ethanol, cocaine, marijuana, and lithium). It is used to diagnose human immunodeficiency virus (HIV), various viral infections, breast and ovarian cancer, oral squamous cell carcinoma, and preterm labour (Lawrence, 2002, Ng et al., 2003) (Table 1-2). Recently published studies suggest that salivary biomarkers may be a practical alternative to serum to diagnose Sjögren's syndrome (SS) (Ching et al., 2011, Hu et al., 2011). Furthermore, saliva testing for Helicobacter pylori has been proven reliable to screen dyspeptic patients (Sonmezoglu et al., 2005). Saliva is also used as a mean for caries risk assessment as well as periodontal inflammatory markers (Wang et al., 2015).

One of the main drawbacks of salivary diagnostics is the low concentrations of analytes found in saliva compared to blood. Despite that fact, Streckfus et al documented human epidermal growth factor receptor 2 (HER2, c-erbB-2) and cancer antigen 15-3 in saliva as the most reliable markers for breast cancer prognosis (Streckfus et al., 2000). Oral cancer patients in comparison to healthy controls, differentially expressed tumour necrosis factor-α (TNFα), interleukin-1 (IL-1), IL-6, IL-8, tissue poly peptide antigen, and cancer antigen 125 which could be potentially useful for early detection (Streckfus et al., 2000). Pancreatic cancer is one of the most lethal malignancies. Zhang et al reported the possibility of salivary biomarkers mRNA (KRAS, CDKL3) to be used for early detection of pancreatic cancer. This however, needs further study due to the modest sample size (n=42) (Zhang et al., 2010).

C-reactive protein (CRP), as reported by Floriano et al, provides an opportunity for non-invasive assessment of cardio-vascular disease (CVD) risk. It needs sensitive detection technologies due to the low concentration in saliva. Further investigations of reference ranges and their correlation with serum concentrations is required (Floriano et al., 2009). Table 1-2 lists some salivary biomarkers used in diagnosis

Table 1-2 Salivary biomarkers in disease diagnosis

Reference	Marker	Disease
(Krishna Prasad et al., 2013)	p53, mRNA	Oral squamous cell carcinoma (SCC)
(Kaufman and Lamster, 2002)	Ca-125	Ovarian cancer
(Kaufman and Lamster, 2002)	IgG	Viral (HIV,HBV,HAV,HCV,EBV,CMV,rubella)
(Streckfus et al., 2000)	C-erb-2, 15-3 antigen	Breast cancer
(Wang et al., 2015)	CRP,IL-1β, IL-6	Periodontitis (inflammatory biomarkers)
(Miller et al., 2010, Floriano et al., 2009)	MYO,CRP,TNFα	Acute myocardial infarction (AMI)

HIV (Human immunodeficiency virus), HBV (Hepatitis B virus), HAV (hepatitis A virus), HCV (Hepatitis C virus), EBV (Epstein-Barr virus), CMV (Cytomegalovirus), CRP (C reactive protein), TNF (Tumour necrosis factor), MYO (Myoglobin), IL (Interleukin)

Saliva has become a more attractive medium due to its minimal potential for cross infection. The collection requires a less invasive procedure, and is also cost-effective. As technology progresses, saliva has the potential to become a first-line diagnostic medium. There is a potential value for it in the diagnosis and management of MMP and PV as investigated to date by, Andreadis et al (27 PV, 12 MMP), Hallaji et al (50 PV) and Mortazavi et al (86 PV) (Andreadis D, 2006, Hallaji et al., 2010, Hosseein Mortazavi, 2015) but these studies need to be confirmed and extended.

1.8 Aims of the Study

This study was designed to look at the clinical and immunpathological findings in MMP and PV in order to address the aims listed below. Patients were drawn from Guy's Hospital Oral Medicine and Dermatology departments as well as Moorfields Eye Hospital to include a broad spectrum of clinical phenotypes.

These aims were intended to relate phenotype to antibody isotypes and target antigens to try to further the understanding of the clinical spectrum and disease severity.

1.8.1 Main aims of the study

1. To investigate the potential of saliva for diagnosis and disease-monitoring in both MMP and PV.

2. To utilize serum and salivary biomarkers in the analysis of disease activity and therapeutic response in both MMP and PV.

3. To investigate whether distinct clinical phenotypes are associated with specific target antigens and epitopes in MMP.

1.8.2 Specific aims of the study

- **Chapter 3**

1) To identify whether serum and saliva IgG and/or IgA autoantibodies to BP180-NC16a were present in our cohort of MMP patients

2) To identify whether there was an association with carefully defined clinical subgroups

3) To utilize serum and salivary biomarkers in the analysis of disease activity and therapeutic responses

4) Expression of the NC16a and 4575 (C-terminal domain) epitopes on BP180 for ELISA testing

- **Chapter 4**

1) To identify the whether serum and saliva IgG and/or IgA autoantibodies to α6β4 were present in our cohort of MMP patients
2) To identify whether there was an association between autoantibodies against α6β4 integrin and carefully defined clinical subgroups
3) To determine antibody avidity using BIAcore technology

- **Chapter 5**

1) To establish whether whole saliva might provide a suitable alternative to serum for diagnosing and monitoring PV

2) To investigate whether anti-Dsg3 IgA antibodies could be detected in serum and saliva

3) To establish whether there was an association between serum or saliva anti-Dsg3 antibodies and disease severity

4) To utilize serum and salivary biomarkers in the analysis of disease activity and therapeutic response

2 Materials and Methods

2 Materials and Methods

2.1 Clinical

2.1.1 Ethical approval

Ethical approval was obtained from the Health research authority REC reference 09/H0721/54 and REC reference 12/LO/1350 (Appendix 1, 2).

2.1.2 Patients

The series consisted of 100 MMP patients, 45 males and 55 females with a ratio of 1:1.2 and a mean age of 65.3 ± (13.0) ranging from (26-89) years. Ninety five of the patients were Caucasians while 5 were either Asian (n=3), African (n=1) or Algerian (n=1). Patients included multisite disease patients (n=51), pure oral (n=33) and pure ocular (n=16). The demographics of the 100 MMP patients are summarised in Appendix 3, as well as details of individual site involvement, oral and ocular severity score, DIF, IIF as well as specific autoantibody titres (IgG, IgA) against BP180-NC16a and α6β4 integrin in serum, whole saliva as well as parotid saliva. In addition, results from our collaborators in Germany, Prof Zillikens, regarding BP180-4575 and Laminin 332 western blot results were obtained.

For PV, the series consisted of 23 PV patients analysed in the PV study plus 3 patients included in the controls for the MMP study. The PV cohort included 10 males and 16 females with a ratio of 1:1.6 and a mean age of 52.1 ± (19.5) ranging from (16-95) years. Fifteen of the PV patients were Caucasians while 10 were Asian and 1 was African. Patients had either mucosal lesions (n=18) or had mucocutaneous lesions (n=8). The demographics of the 23 PV patients as well as site involvement, oral severity score, DIF, IIF, Dsg1 ELISA and specific autoantibody titres (IgG, IgA) against Dsg3 are summarised in Appendix 4.

Patients admitted to the studies were drawn from those attending clinics at Guy's and St. Thomas's Hospital Oral Medicine department (GSTT NHS Foundation Trust), Dermatology department and Moorfields Eye Hospital. Patients were diagnosed with either MMP or PV on the basis of their clinical presentation, direct immunofluorescence (DIF), indirect immunofluorescence (IIF) and classical histopathology. A total of 100 MMP patients, 26 PV patients, 50 healthy controls (HC) and 16 diseases controls (DC) with lichen planus (LP) were recruited. LP patients were included based on the clinical presentation, histology and negative DIF and IIF.

Three studies were conducted to investigate IgG and IgA autoantibody in serum and saliva reactivity to specific autoantigens in MMP and PV patients: Table 2-1 summarises the distribution of patients between the different studies described in this thesis.

Table 2-1 Summary of the distribution of patients between the different studies described in this thesis

Study name	Patients	Disease controls (total)		Healthy Controls
	MMP			
MMP studies: -Study 1(a) (NC16a)	78	PV	LP	50
		6	10 (16)	
-Study 1(b) (NC16a)	22	8	10 (18)	18
-Study 2 (α6β4)	100	21	15 (36)	40
PV study	PV	MMP	LP	17
	23	8	11 (19)	

Total number of patients recruited for all studies	MMP	PV	LP	HC
	100	26	16	50

MMP= mucous membrane pemphigoid, PV= pemphigus vulgaris, LP= lichen planus, DC= disease control, HC= healthy control.

Studies include:

1) Study 1: Serum and salivary antibodies to BP180-NC16a in patients with Mucous Membrane Pemphigoid

a) Study 1(a) in relation to clinical phenotype and disease activity (Cross-sectional study): Use of ELISA to test serum and whole saliva activity against BP180-NC16a in a cross-sectional study. This study included MMP patients (n=78), HC (n=50) and DC (n=16) comprising patients with PV (n=6) and LP (n=10). MMP patients included 37 males and 41 females with a 1:1.1 ratio and a mean (± standard deviation) age of 65.5 (± 11.6) years with a range (26-87 years). Twenty randomly selected MMP patients (20/78) provided parotid saliva as well as 6/50 of the HC. All PV and LP patients provided parotid samples. DIF was positive in 59/77 (77%) of the patients excluding one patient with missing DIF, and 23/65 (35%) having a positive IIF excluding patients with missing IIF results (13/78) so only 8 patients had both DIF and IIF negative results. The majority of patients were on systemic immunosuppressive and immune-modulating treatment that included prednisolone, dapsone, sulfamethoxypyridazine, azathioprine or mycophenolate mofetil. Some were on topical treatment such as corticosteroid eye drops or betamethasone mouthwashes (0.5 mg) in 10 mL water. Patients had been on treatment for an average of 9.2 ± 4.5 years range (2-20) years prior to inclusion in the study. Disease controls included 4 males and 12 females with a ratio of 1:3 and a mean age of 47.0 (±12.1) years. Healthy controls consisted of 24 males and 26 females with a ratio of 1:1.1 and a mean age of 60.5 (±12.9) years.

b) Study 1(b) in relation to therapeutic response (Longitudinal study): Use of ELISA to test serum and whole saliva activity against BP180-NC16a in a longitudinal study. This study included a total of 22 MMP patients in addition to HC (n=18) and DC (8 PV, 10 LP). Samples were collected at 3-month intervals for 9 months. The mean age of MMP patients was 65.7 (±15.3) with a range of (29-89) years. Ten males and 12 females were included with a ratio of 1:1.2. DIF was positive in 21/22 (95%) patients. IIF was positive in 11/22 (50%) of the patients with only one patient having a negative DIF and IIF result. Patients were on treatment for an average of 5.9 (±0.9) years with a range of (2-18) years at the start of the study. Disease controls included 6 males and 12 females with a ratio of 1:2 and a mean age of 49.7 (±18.3). Healthy controls included 6

males and 12 females with a ratio of 1:2 and a mean age of 59.4 (±10.4) years. Parotid saliva was provided by all MMP and DC. Six HC provided parotid saliva.

2) Study 2: **Serum and salivary antibodies to alpha 6 beta 4 Integrin in relation to clinical phenotype and disease activity (Cross-sectional study)**

MMP ELISA testing serum, whole and parotid saliva against α6β4 integrin (cross-sectional) included MMP patients (n=100), HC (n=40) and DC (n=36) (PV=21 and LP= 15). MMP patients consisted of 45 males and 55 females with a ratio of 1:1.2 and a mean age of 65.3 (±13.03) ranging from (26-89). DIF was positive in 76/94 (81%) and IIF was positive in 36/87 (40%) of the patients excluding those with missing IIF results (13/100). Nine patients had a negative result on both DIF and IIF. Disease controls involved 12 males and 24 females with a ratio of 1:2 and a mean age of 49.6 (±17.9). Healthy controls included 19 males and 21 females with a ratio of 1:1.1 and a mean age of 61.3 (±11.4). Parotid saliva was provided by 40/100 MMP patients, 6/40 HC and all DC.

3) PV study: Serum and salivary antibodies to Dsg3 in relation to disease activity (Cross-sectional) and Sequential antibody titres in PV patients to Dsg 3 in relation to therapeutic response (Longitudinal study)

Activity of serum as well as whole and parotid saliva against Dsg3 was determined by ELISA. This study included PV patients (n=23), HC (n=17) and DC (n=19) (8 MMP and 11 LP). PV patients comprised 10 males and 13 females with a ratio of 1:1.3 and a mean age of 51.1 (±19.2) ranging from (16-95). Inclusion criteria were that patients had predominantly mucosal involvement and at least one of either positive direct immunofluorescence (DIF) (23/23 100%), positive indirect immunofluorescence (IIF) (17/23 74%) and / or a positive ELISA with commercially available plates for Dsg 3(17/23 74%). All patients were on a combination of prednisolone and azathioprine or mycophenolate mofetil as well as a betamethasone mouthwash. Patients were on treatment for an average of 5.4 (±4.3) years (range 2-22 years). Disease controls were 6 males and 13 females with a ratio of 1:2.2 and a mean age of 55.3(±15.8) years. Healthy controls included 6 males and 11 females with a ratio of 1:1.8 and a mean age of 58.9 (±10.4) years. Both PV and DC provided parotid saliva. Six of the HC provided parotid saliva. Samples were collected at 3-month intervals for 9 months.

2.1.3 Sample Collection

Matched serum and whole saliva samples were collected from all participants including MMP, PV, LP and HC. In addition, parotid saliva was obtained from 40 of the MMP

patients (n = 40/100), all PV (n = 26) and LP (n = 16) patients and 6 of the HC (n = 6/50).

2.1.3.1 Serum

Blood samples were collected in 5 ml tubes in the clinic by a member of the trained clinical staff. Specimens were then taken to the lab with a name and study ID. A tracker sheet indicating date, time and volume was completed. Serum was obtained by centrifugation at 5000 g for 10 minutes (Mistral 3000 MSE Ltd, London, UK). Serum was stored as aliquots (0.5 mL) at -80°C for long term storage. Samples for immediate testing were kept at -20° C.

2.1.3.2 Saliva

Whole un-stimulated saliva was collected in 20 mL universal tubes and patients were asked to spit saliva in the tube for a 10 minute period as previously described (Percival et al., 1994). Parotid saliva was first stimulated with a few drops of 2% citric acid just at the beginning of the collection and the saliva was then collected into a universal container as previously described (Challacombe, 1976). The collection was done by a commercially available Lashley cup which is routinely used for the collection of parotid saliva in the clinic. It is a small plastic cup of two concentric rings with a tube out of each, one to be able to apply mild suction to hold the cup in place, and the other allows the parotid saliva to flow directly to a collection bottle. Whole saliva was centrifuged at 3000 g for 5 minutes. All samples were aliquoted (0.5 ml) and stored at -80° C. The aliquots subsequently used in this study were then all stored at -20° C.

2.1.4 Clinicopathological associations

Patients with MMP were examined by specialists in each area (Dermatology, Oral Medicine, Otolaryngology, Ophthalmology) carefully to determine the presence and extent of disease in each potentially affected site (mouth, nasopharynx, larynx, conjunctiva, skin). A previously described Oral diseases scoring methodology for use in lichen planus and modified from a published methodology for MMP was used (Escudier et al., 2007) (Appendix 5).

Patients with PV were also assessed for severity of oral disease using the same methodology. For the longitudinal studies, serial oral severity scores were undertaken at each visit. Clinicopathological associations could then be analysed for these studies.

2.2 Laboratory Techniques

List of antibodies and dilutions used: Dilutions of antibodies used in ELISA were derived from previous work in our lab (data not shown) while those for Western Blots were used as per manufacturer's instructions. (List of Buffers and reagents is described in Appendix 6)

- Alkaline phosphatase conjugated goat polyclonal anti-human IgA antibody (A3400, SIGMA-ALDRICH) (1:500 / 1:1000)
- Mouse monoclonal anti-human secretory component antibody (I6635, SIGMA-ALDRICH) (1:1000)
- Rabbit polyclonal anti-mouse IgG antibody alkaline phosphatase linked (A1902, SIGMA-ALDRICH) (1:1000)
- Rabbit polyclonal anti-human IgA (Dakopatts A0262) (1:10000)
- Goat anti-human-IgA (2010-04 SouthernBiotech) (1:1000)
- Rabbit polyclonal anti-goat IgG alkaline phosphatase linked (A4062, SIGMA-ALDRICH) (1:1000)
- Goat polyclonal anti-human IgG (A8542, SIGMA-ALDRICH) (1:500)
- Goat polyclonal anti-human IgM (A3437, SIGMA-ALDRICH) (1:500)
- Mouse monoclonal anti-human IgA antibody (SkyBio M26012) (1:1000)
- Rabbit polyclonal anti-mouse IgG peroxidase antibody (A9044 SIGMA-ALDRICH) (1:2500)
- Goat polyclonal anti-human IgG human integrin alpha 6/CD49f antibody (AF1350, R&D systems) (1:500)
- Sheep polyclonal anti-human IgG human integrin beta 4/CD104 antibody (AF4060 R&D systems) (1:1000)
- Donkey polyclonal anti-sheep IgG alkaline phosphatase (A5187, SIGMA-ALDRICH) (1:1000)
- Rabbit polyclonal anti-goat IgG alkaline phosphatase (A4187, SIGMA-ALDRICH) (1:1000)

2.2.1 ELISA

2.2.1.1 Analyses of antibody binding activity to the NC16a domain of BP180

Commercially available pre-coated plates with the NC16a domain of BP180 (MESACUP BP180 ELISA Kit, Medical and Biological laboratories Nagoya, Japan. Woburn, MA 01801) were used to test for both specific IgG and IgA antibody in serum as well as in whole and parotid saliva (Fig 2-1).

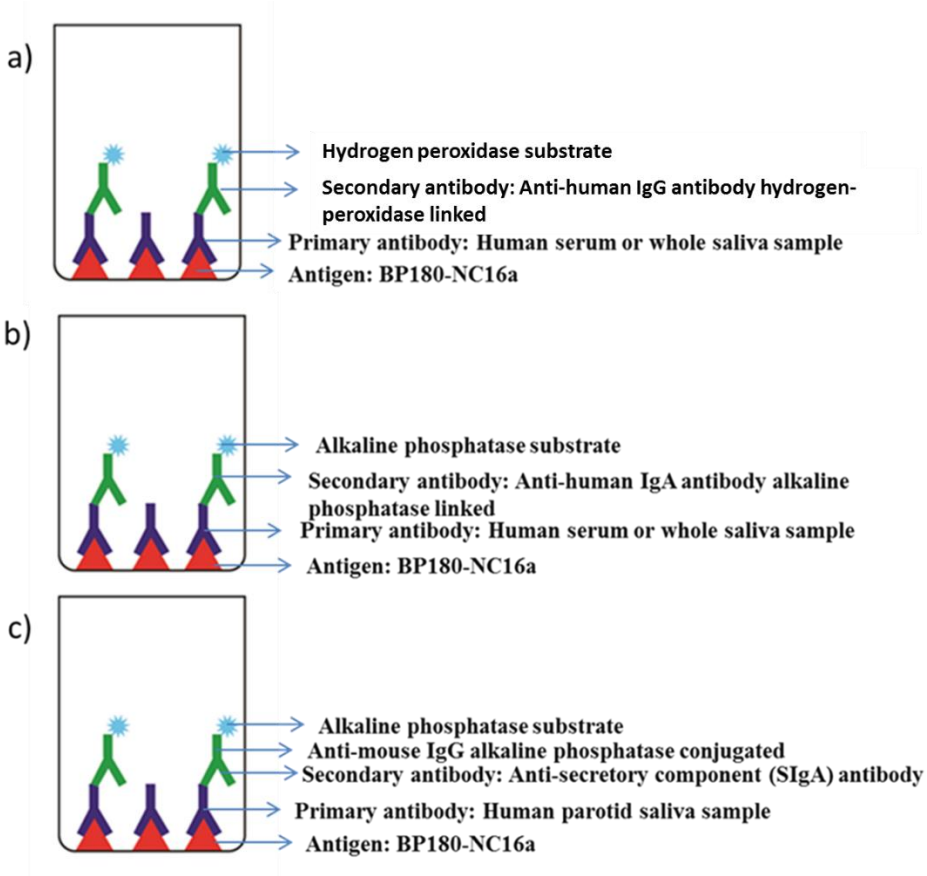


Figure 2-1 BP18-NC16a ELISA plates. ELISA wells describing the different layers used. a) Testing Human serum or whole saliva samples for IgG antibody to BP180-NC16a. b) Testing Human serum or whole saliva samples for IgA antibody to BP180-NC16a. c) Testing Human parotid saliva samples for IgA antibody to BP180-NC16a

2.2.1.1.1 Analysis of IgG antibody against BP180-NC16a

The plates were optimized for serum IgG antibody testing and the manufacturer's instructions were followed. All incubations were carried out at 37° C in the (Mini/4/SS/VS scientific supplies, Nottingham UK). Calibrators (a positive and a negative control were provided with the kit) and patient sera (10 fold dilution) were added to these plates allowing anti-BP180 antibodies to react with the antigen for 1 hour. After washing with PBS/Tween 20 (3 times) using the LT-3500 microplate washer (Labtech East Sussex UK) to remove unbound serum proteins, horseradish peroxidase conjugated anti-human IgG antibody was added and plates were incubated for 1 hour (Conjugate incubation). After further washing with PBS/Tween 20, the peroxidase substrate, tetramethylbenzidine dihydrochloride/hydrogen peroxide (TMB/H_2O_2) (supplied with the kit), was added and plates were incubated for 30 minutes (substrate incubation) as shown in (Fig 2-1). 1.0N sulphuric acid was then added to terminate the reaction and stabilize colour development. Bound antibody was determined by measurement of OD_{450nm}. This was quantified by measuring the reaction photo-metrically. All materials were supplied with the kit. This has been described previously (Sakuma-Oyama et al., 2004).

In order to test for reactivity for IgG antibody in whole saliva, samples were incubated for 1 hr and the manufacturer's conjugate and substrate were used. The sensitivity of detection of specific IgG antibody in saliva was determined separately. Using the pre-coated BP180-NC16a plates, the positive pooled sera standard was spiked with PBS (50 µl), whole saliva containing IgG-/IgA+ antibody against BP180-NC16a or whole saliva negative for both IgG and IgA antibody against BP180-NC16a. The samples were tested in duplicates in 7 doubling dilutions (1:100, 200, 400, 800, 1000, 3200 and 6400). Otherwise, conditions were those recommended by the manufacturer, as above.

2.2.1.1.2 Analysis of IgA antibody against BP180-NC16a

Incubation times for anti-human IgA antibody were previously optimized in this laboratory (Coogan et al., 1994). Alkaline phosphatase conjugated goat polyclonal anti-human IgA antibody was used to detect bound antibody (A3400, SIGMA-ALDRICH). Phosphatase substrate (4-nitrophenyl phosphate disodium salt hexa-hydrate) in tablet form was dissolved in 1M diethanolamine pH 9.8, and 3M NAOH (50 µl) was used to stop the reaction. Both the sample and conjugate incubation times were 2 hours at 37° C and the assay diluent (PBS/ Tween 20 and 0.09% sodium azide) provided with the kit was used.

To determine whether IgA in parotid saliva samples that bound to the NC16a domain, was secretory form, bound antibody was probed with mouse monoclonal anti-human secretory component antibody (I6635, SIGMA-ALDRICH) using rabbit polyclonal anti-mouse IgG antibody alkaline phosphatase linked for detection (A1902, SIGMA-ALDRICH). In parallel, plates were also probed with the conjugated goat anti-human IgA as above. Incubations with saliva and the anti-secretory component antibody were both for 2 hours at 37° C. Incubation with the alkaline phosphatase-conjugated antibody was for 1 hour at 37° C as shown in (Fig 2-1). Phosphatase substrate was added and the reaction was stopped with 3M NaOH. For parotid samples, the results were expressed as OD_{405nm} values since antibody levels and background were much lower and fell below values derived from a standard curve from serum, they could not be quantified using the serum derived standard curve.

The specificity of the monoclonal anti-secretory component antibody, used above, was confirmed by ELISA

A) - A 96-well-plate (Immulon 2HB Thermo lab systems), divided into three sections each coated with a Rabbit polyclonal anti-human IgA at different dilutions; 1:5000, 1:10000, 1:20000 in Phosphate buffered saline (PBS) for a checkerboard optimization of coating. The optimal coating concentration was found to be 1:10000. The plate was then blocked with PBS/ BSA (Bovine serum albumin)/ Tween20 (P1379 TWEEN (R) 20 SIGMA-ALDRICH) for 1 hour at 37° C. All dilutions were done in PBS/ BSA/ Tween 20 and all remaining incubations were at 37° C. Three serum samples were tested in duplicate at 6 doubling dilutions (1:100, 200, 400, 800, 1600, and 3200) and incubated for 2 hours. The secondary antibodies added were goat anti-human-IgA (A3400, SIGMA-ALDRICH) and another using the mouse anti-human monoclonal anti-secretory component antibody for 2 hours. The conjugates used were anti-mouse IgG alkaline phosphatase linked (A1902, SIGMA-ALDRICH) and an anti-goat IgG alkaline phosphatase (A4062, SIGMA-ALDRICH) linked incubated for 1 hour. The phosphatase substrate and stop solution were used and results were reported in $OD_{405\ nm}$.

B) - Parotid saliva samples were tested using the same pre-coated BP180-NC16a plates and the mouse monoclonal anti-secretory component (IgA) antibody with different conjugates. In this experiment, a goat polyclonal anti-human IgG

(A8542, SIGMA-ALDRICH) and a goat polyclonal anti-human IgM (A3437, SIGMA-ALDRICH) were used under the same conditions described for the parotid saliva testing.

2.2.1.1.3 Quantitation of anti-NC16a antibody

Optical density (OD) was measured at 450 nm for IgG and 405 nm for IgA using an ELISA plate reader (LT4000 Labtech microplate reader). All samples were screened for both IgG and IgA specific antibody and a standard was developed using a range of pooled antibody (both IgG and IgA) positive serum samples. The standard was given an arbitrary value of 100,000 units and standard curves constructed using 6 doubling dilutions starting at 1:100 as previously described (Challacombe, 1976). Serum IgG, IgA, whole saliva IgG and IgA antibody titres were expressed in units by calculating the mean of values of four doubling dilutions falling on the reference curve. The starting dilution for serum samples was 100 fold dilution while a 10 fold dilution was used for whole saliva. The 1:10 dilution was found to offer a better sensitivity than 1:50 shown in Appendix 7.

The reproducibility of the ELISA was measured by the coefficient of variance value. A positive control sample used in each plate for all variables; a positive serum sample for serum IgG and serum IgA and a positive saliva sample for saliva IgG and saliva IgA, as well as a positive parotid sample for parotid IgA and SIgA. The samples were used in each plate to calculate the reproducibility of each plate (Intra and inter-plate variation). A total of 20 plates were calculated. ELISA mean coefficient of variation for intra-plate variation of serum IgG antibodies was 9.6% and for IgA antibodies was 10.4%. Inter-plate variation for serum antibodies for IgG was 11.4% while it was 12% for IgA. Assay of whole salivary antibodies was more variable with a coefficient of variation up to 18%. Parotid saliva had a coefficient of variation of 16% (Table 1-2).

Collaborator's results:

Our collaborators D. Zillikens and E. Schmidt from Germany were kind enough to share some of their results. They tested 39 samples from our MMP cohort for IgG and IgA antibody reactivity to the 4575 epitope of BP180 and to laminin 332. These results are shown in Appendix 3.

Table 2-2 The mean coefficient of variation for ELISA tests

	Serum IgG	Serum IgA	Salivary IgG	Salivary IgA	Parotid IgA	Parotid SIgA
Intra-plate coefficient of variance	9.6%	10.4%	15.6%	17.5%	14.5%	15.9%
Inter-plate coefficient of variance	11.4%	12%	16.4%	18.2%	15.6%	16.02%

Size-exclusion chromatography of whole saliva

Five whole saliva samples positive (n = 4) or negative (n = 1) for anti- BP180-NC16a IgA antibody, as determined by ELISA using the anti-secretory monoclonal antibody for detection, were fractionated by size exclusion chromatography. The aim of this experiment was to determine whether anti-BP180-NC16a binding activity was associated with high molecular weight SIgA. Saliva (125 µl 1:2 dilution in 100mM (tris (hydroxymethyl) aminomethane, THAM)-HCL pH 8.0) was loaded on a Sephacryl S-200 column (10/300, GE Healthcare) and chromatography was performed using the ÄKTAbasic system (GE Healthcare). The elution buffer was 10 mM ammonium bicarbonate. Absorbance was monitored at 280 nm and 0.5 ml fractions were collected at a flow rate of 0.5 ml/min. Fractions were lyophilized (Edwards freeze dryer) and re-suspended in 400 µl of PBS. Fractions of defined molecular weight were analysed by Western Blotting (as described below) for the presence of secretory component and IgA. Fractions that included secretory component and IgA were further tested for binding activity against BP180-NC16a using the pre-coated ELISA plates as above. The Sephacryl S-200 column was calibrated by chromatography of molecular weight standards: apoferritin (480 Kda), gamma globulin (120 Kda), ovalbumin (45 Kda) and cytochrome C (12 Kda).

Denaturing Polyacrylamide Gel Electrophoresis

1) - NuPAGE® Novex® 4-12% Bis-Tris Protein Gels, 1.0 mm, with either 15 wells (NP0323 Life Technologies) or 10 wells (NP0321 Life Technologies) were used to separate proteins according to molecular weight. ColorBurst (TM) Electrophoresis Marker, mol wt 8,000-220,000 Da (C1992-1VL Sigma-Aldrich) was loaded. Samples were prepared as recommended by addition of NuPAGE® LDS (lithium dodecyl sulphate) sample buffer (4X concentrated) followed by heating at 100°C for two minutes. Electrophoresis was at 120 V 150 mA (EPS00 Pharmacia) for 50 minutes using the NuPAGE® MOPS SDS Running Buffer (for Bis-Tris Gels only) (20X) (NP0001 Invitrogen). For electrophoresis under reducing conditions, samples were heated in the presence of 0.1M DTT (dithiothreitol).

Proteins were visualised by incubating gels in 0.25% (w/v) Comassie brilliant blue R250 in 25% (v/v) methanol, 10% (v/v) acetic acid in distilled H_2O followed by destaining of background with 25% methanol, 10% acetic acid in distilled H_2O.

Western blotting

For Western blotting, proteins were transferred to nitrocellulose membranes using the wet transfer protocol (Power pack basic, Bio-rad, Hertfordshire UK) with 1XTransfer buffer at constant current (400 mA) with maximum of 90 V for 90 minutes. After the transfer was complete the gel was stained with Coomassie blue as above to confirm protein transfer.

Membranes were blocked by incubation in 5% milk (dry milk powder) in PBS for 1 hour at room temperature on a shaker (SSM3 mini gyro-rocker, BioCote Coventry UK) and were washed with PBS/ Tween 20, 3 times (3x) 10 minutes each. Primary antibody in 2% milk in PBS was then added and membranes were incubated with gentle shaking overnight at 4° C (SSM1 mini orbital shaker, BioCote Coventry UK). Membranes were then washed 3x 10 minutes with PBS/ Tween 20. The secondary antibody (horse radish peroxidase conjugate) in 2% milk in PBS was then added and membranes were incubated for 1 hr at room temperature with gentle shaking. Membranes were again washed 3x times with PBS/Tween 20 and peroxidase substrate (Luminata Crescendo Western HRP Substrate WBLUR0500 Millipore) was then added. Membranes were incubated for 90 seconds following the manufacturer's instructions. The film (47410 19236 Fuji medical x-ray film) was then developed using an automated x-ray film processor (Model JP-33 Jpi) at different time exposures for optimal image.

2.2.1.2 α6β4 Integrin Coated Plates

Recombinant human integrin alpha 6 beta 4 (α6β4) 50 µg (5497-A6-050 R&D systems) was reconstituted as per manufacturer's instructions. This was used to coat a 96-well ELISA plate at different concentrations to obtain the optimal coating concentration for testing the samples. A checkerboard test was designed; 0.1, 0.5 and 1 µg/ml α6β4 in carbonate/ bicarbonate 9.6 pH buffer was used to coat the plate which was then left incubating at 4°C overnight. All incubations thereafter were at 37° C. The plate was then allowed to reach room temperature and blocked for 1 hr with PBS/BSA/T20 which was then used as a diluent for the assay. Anti-sera, both α6 and β4 (1:250, 500 and 1000) at different concentrations were added and incubated for 2 hours. These were Goat anti-human polyclonal IgG human integrin alpha 6/CD49f antibody (AF1350 R&D systems) and sheep anti-human polyclonal IgG human integrin beta 4/CD104 antibody (AF4060 R&D systems). The secondary antibodies were alkaline phosphatase conjugated at a 1:1000 dilution and incubated for 1 hr. Donkey anti-sheep polyclonal IgG alkaline phosphatase (A5187 SIGMA-ALDRICH) and rabbit anti-goat polyclonal IgG alkaline phosphatase (A4187 SIGMA-ALDRICH) were used respectively. The previously mentioned alkaline phosphatase substrate was used to develop the plate which was incubated for 20 minutes stopped with 3M NaOH and $OD_{405\ nm}$.

The optimal concentration was found to be 0.1 µg/ml and all subsequent plates were coated with that concentration (Appendix 8). Serum and saliva samples were screened at 1:50 and 1:10 respectively to obtain a positive sample used as the positive control for all plates. All samples were tested in duplicates. Antibody IgG and IgA against α6β4 integrin was then tested for in serum and saliva using an anti-human polyclonal IgG alkaline phosphatase linked (A8542 SIGMA-ALDRICH) and an anti-human polyclonal IgA alkaline phosphatase linked (A3400 SIGMA-ALDRICH) at a 1:500 dilution. Incubations were at 37° C for 2 hrs. In between steps, the plates were washed with PBS/ T 20. A positive sample was used repeatedly in order to establish the mean coefficient of variation for serum and saliva plates (a total of 10 plates used); IgG antibody in serum (Intra-plate) was 10% while inter-plate variation was 13%. IgG antibody saliva intra-plate variation was 13.5% while inter-plate variation 15%.

2.2.1.3 Dsg3 Pre-coated Plates

Commercially available pre-coated plates with the desmoglein 3 antigen (Dsg3) MESACUP Dsg3 test (Medical and Biological laboratories Nagoya, Japan) were used to test for both IgG and IgA antibody to Dsg3 in serum, whole and parotid saliva. All

samples were screened for both IgG and IgA anti-Dsg3 antibody and a standard was developed using a range of pooled antibody (both IgG and IgA) positive serum samples. The standard was given an arbitrary value of 100,000 units and standard curves constructed using 6 doubling dilutions starting at 1:100 as previously described (Challacombe, 1976). Serum IgG, IgA, whole saliva IgG and IgA antibody titres were expressed in units by calculating the mean of values of four doubling dilutions falling on the reference curve reported at OD_{450} nm. The starting dilution for serum samples was 100 fold dilution while a 10 fold dilution was used for whole saliva. Measurement of serum IgG antibody against Dsg3 was performed according to the manufacturer's instructions using reagents provided with the kit. This has been previously described (Ishii et al., 1997). Optimal conditions were established by screening all samples in duplicates (serum 1:50 and saliva 1:10) with different conjugate concentrations and incubation times for anti-Dsg3 IgG antibody in saliva and anti-Dsg3 IgA antibody in serum and saliva. Serum and saliva samples (1:100 dilution for serum and 1:10 for saliva) were tested for IgA antibody against Dsg3 as described for BP180-NC16a except that the anti-IgA antibody was used at a dilution of 1:1000 and incubations were for 1 hour instead of 2 hours at OD_{405nm} (Appendix 9). The mean coefficient of variation was calculated by using a positive sample repeatedly in each plate. The mean coefficient of variance (intra-plate) for IgG antibody in serum was 9.4% and inter-plate variation was 10%. The mean coefficient of variance (intra-plate) for IgA antibody in serum was 10.6% and inter-plate variation was 12.1%. The mean coefficient of variance for IgG antibody in saliva (intra-plate) was 11.2% while inter-plate was 13%.

Dsg1 results:

The Dsg1 results used in this thesis were completed by Mr.Bhogal at the St John's Institute of Dermatology, Guy's and St Thomas's NHS Foundation Trust, London, United Kingdom.

2.2.2 Expression and purification of recombinant BP180

Plasmids:

pGEX2T was from GE Healthcare Life Sciences Buckinghamshire UK.

E.coli BL21strains harbouring recombinant pGEX2T encoding fusion proteins with the NC16a epitope (residues 490-562 of BP180) and the 4575 epitope (residues 1365-1413 of BP180) were kindly provided by D. Zillikens and E. Schmidt (University of Luebeck, Germany)

pET15b was from MerkMillipore Hertfordshire UK (Cat. No 6966-13).

Bacterial cells:

E. coli XL1 Blue: genotype {recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacIqZΔM15 Tn10 (Tetr)], used for DNA production, was from Stratagen Santa Clara, CA United states (Cat. n. 200249).

E. coli BL21 (DE3): genotype [F–ompT hsdS (rB–mB–) dcm+ Tetr gal λ (DE3); used for protein expression Stratagene], used for protein production was from Stratagene (Cat. no. 230280).

Bacterial Media:

LB (Lysogeny Broth) medium: 10g/L Tryptone (Oxoid, Cambridge, UK); 5g/L Yeast extract (Oxoid, Cambridge, UK); 10g/L NaCl (Sigma-Aldrich, Dorset, UK).

LB Agar: 15g/L Agar Bacteriological (Oxoid, Cambridge, UK) dissolved in LB medium.

SOC medium: 20g/L Tryptone (Oxoid, Cambridge, UK); 5g/L Yeast extract (Oxoid, Cambridge, UK); 0.5 g/L NaCl; 4.8 g/L MgSO4 (Sigma-Aldrich, Dorset, UK).

2.2.2.1 Purification of plasmid DNA

Plasmid DNA was purified from 1 - 5 ml cultures, grown overnight in LB supplemented with carbenicillin (50µg/ml) using the QIAprep Spin Miniprep Kit (QIAGEN, West Sussex, UK) according to the manufacturer's instructions. The system is based on alkaline lysis of bacterial cells. *E.coli* cells were harvested by centrifugation and re-suspended in 10 mM EDTA, 100 µg/ml RNase A in 50 mM Tris-HCl (pH 8.0). Lysis buffer (200mM NaOH, 1% SDS) was added followed by 3M KAcetate. Precipitated cell debris and denatured chromosomal DNA were removed by centrifugation and the supernatant was applied to silica resin. Following washing with ethanol for further removal of RNA, proteins and salts, plasmid DNA was eluted in 50 µl of deionized water or TE buffer.

The concentration of purified DNA was determined using the Nanodrop 2000 (Labtech International Ltd, East Sussex, UK). DNA preparations with an $OD_{260\ nm}/OD_{280\ nm}$ ratio in the range 1.8-2.0 (indicating low level contamination with protein) were suitable for further use.

Digestion of DNA with restriction enzymes:

Restriction enzymes were all from New England Biolabs and were used according to the manufacturer's instructions. Generally 10-20 U of restriction enzyme were used to digest 1 µg of plasmid DNA in the presence of the buffer supplied. Mixtures were incubated at 37°C for a minimum of 60 minutes. Enzyme inactivation, when necessary, was carried out after digestion by heating the reaction for the time and temperature required for each enzyme according to the manufacturer's instructions. DNA digestion was verified by agarose gel electrophoresis.

Ligation of DNA:

Purified plasmids (approximately 100 ng), linearised by restriction enzyme digestion, were combined with a 3-fold molar excess of insert DNA in the presence of the supplied ATP-containing ligase buffer (30mM TRIS-HCl pH 7.8, 10mM MgCl2, 10mM DTT and 1mM ATP) and 10 U (1 µl) of T4 DNA ligase. The reaction was incubated for 2 hours at 25 °C, temperature at which the enzyme has optimal activity. Control reactions with no insert or no polymerase were included to indicate the level of non-recombinant transformants (see below).

Polymerase Chain Reaction (PCR):

DNA amplification was performed by PCR with plasmid DNA (10ng) as template. The reaction mixture (total volume 50µl) also contained dNTPs (deoxyribonucleotides: dATP, dCTP, dGTP and dTTP) each at 0.2 mM, 2.5 mM $MgCl_2$ (Promega, Southampton, UK), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 0.1% (v/v) Triton X-100, 0.1 mg/ml BSA in 200 mM Tris-HCl (pH 8.8 at 25°C) with 2.5U PfuUltra™ HF DNA polymerase (Stratagene, La Jolla, CA, USA); 10pmol each of forward (sense) and reverse (antisense) oligonucleotides were included as primers (Sigma-Aldrich, Dorset, UK). Following initial template denaturation (2 min at 95°C), DNA was amplified by 30 denaturation-annealing-extension cycles. Finally, reactions were incubated for 1 min at the annealing temperature and for 5 min at 72°C for final annealing and extension. Extension time was evaluated according to the size (BP) of the target DNA. Annealing conditions were optimized based on the oligonucleotide sequences.

2.2.2.2 Preparation of electrocompetent *E.Coli*

LB medium (5 ml) was inoculated with *E. coli* (single colony from agar plate) and incubated for 16h at 37°C with shaking at 250 rpm. The culture was then used to inoculate 500ml of LB medium in a 2 l-conical flask. Incubation was at 37°C with

shaking at 250 rpm until the OD_{600nm} of the culture was 0.5. The culture was then cooled on ice for 30 min with swirling of the flask every 5-10 min. Bacterial cells were harvested by centrifugation at 150 x g for 20 min at 4°. The cell pellet was re-suspended in sterile ice-cold distilled water and cells were again recovered by centrifugation at 150 x g for 20 min at 4°C. The cell pellet was gently re-suspended in 50 ml of sterile ice-cold distilled water and transferred to 50-ml polypropylene tubes. Cells were again recovered by centrifugation (720g, 20min, and 4°C) and re-suspended in 20ml ice-cold 10% glycerol (in H_2O). Following further centrifugation (720g, 20min, 4°C), cells were re-suspended in 2 ml of 10% glycerol and aliquoted (100 µl). Aliquoted electrocompetent cells were frozen in liquid nitrogen and stored at -80°C.

Transformation of *E.coli* by electroporation:

DNA (either purified plasmids or ligation reaction mixture) was desalted to reduce the likelihood of sample arcing using the QIAquick PCR purification Kit (QIAGEN) according to the manufacturer's instructions and was eluted in deionized water. Electrocompetent *E.coli* were thawed on ice 15 min before transformation. Cells (45 µl) were transferred to an ice-cold electroporation cuvette (gap width 0.1 cm) (Biorad CA, USA) and gently mixed with the desalted DNA (1-10 ng in a maximum of 10 µl). Electroporation was carried using the Gene-Pulser (Biorad) set to discharge a 25 uF capacitor, charged to 2.5kV in parallel with a 200 Ω resistor giving time constants ranging between 4.5 and 5 m/s. SOC medium (950 µl) was added immediately after electroporation. The cell suspension was then incubated for 60 min at 37°C with shaking at 250 rpm to allow for expression of the antibiotic resistance gene. Cells were then plated on LB agarose plates supplemented with carbenicillin (50 µg/ml).

Screening of transformants:

LB (3 – 5ml) supplemented with carbenicillin (50 µg/ml) was inoculated with selected transformants and cultures were incubated at 37˚ C for 16 hours. For some cultures, isopropyl β-D-1-thiogalactopyranoside (IPTG, 1mM) was added to induce expression of recombinant protein. Aliquots (1 ml) of cultures were frozen in 20% (w/v) glycerol and stored at -80°C. Cells were recovered by centrifugation and resuspended for plasmid purification. Purified plasmids were screened for the presence of inserted DNA by restriction enzyme digestion or polymerase chain reaction (PCR) followed by agarose gel electrophoresis.

For analysis of protein expression, cells were lysed. Lysates were resolved by SDS-PAGE and expression of recombinant GST-fusion protein was determined by western blotting using anti-GST antibody.

DNA agarose gel electrophoresis:

DNA plasmids or fragments were analysed by agarose gel electrophoresis. Gels were prepared by dissolving electrophoresis grade agarose [(0.8-2% w/v) Sigma-Aldrich, Dorset, UK] in TAE buffer (0.04M Tris-acetate, pH 8.0; 1 mM EDTA). The intercalating nucleic acid stain GelRed (Biotium, Hayward, CA, USA) was added (1 in 10^4 dilution) to the solution after it was cooled to 55-60 °C. The gel solution was poured into a cast with the sample well comb inserted. DNA samples were mixed with loading buffer [0.04% (w/v) bromophenol blue; 0.04% (w/v) xylene cyanol; 5% glycerol] and loaded into wells at the end of the gel closer to the anode. A voltage of 80-120 V was applied allowing for DNA migration to the positive terminal (cathode). Bromophenol blue and xylene cyanol served as tracking dyes migrating at about the same rate as a 500 or 4000 base pair DNA fragment, respectively. DNA molecular weight markers (1 kb DNA ladder, 100bp DNA ladder; New England Biolabs, Ispwich, MA, USA) were run in parallel with the DNA samples. DNA fragments were visualised by UV light exposure. Pictures were taken by using a gel documentation system (Syngene).

Preparative agarose gel electrophoresis for purification of DNA fragments:

For sub-cloning, digested plasmid and inserts were resolved on agarose gels and recovered from excised gel slices. Agarose gels were prepared as described above with autoclaved TAE buffer. With the exception of the chamber lid, all the equipment used for electrophoresis (chamber, gel cast and combs) was pre-washed with 10% SDS and rinsed with autoclaved distilled water in order to minimise nuclease activity. DNA (1-5 µg) in 20-60 µl was applied to the gel wells. Electrophoresis was at 80 volts for 4-5 hours for optimal separation. DNA was visualised by brief (5 – 10s) exposure to long wavelength UV to reduce the likelihood of phosphodiester bond cleavage (DNA nicking). DNA bands corresponding to inserts and linearized vectors were excised with sterile blades and purified using the QIAquick Gel Extraction Kit (QIAGEN) following the manufacturer's instructions. This system is based on DNA selective absorption by a silica membrane. Agarose gel slices were solubilized using a guanidine thiocyanate-containing buffer which also provided the high-salt concentration conditions required for DNA binding to the silica membrane. Following adsorption to silica and washing

with 70% ethanol, DNA was eluted in 15-30 µl of deionized water or TE buffer (10 mM Tris pH 8.0, 1mM EDTA).

2.2.2.3 Sub-cloning of NC16a-GST and 4575-GST in pET15b

DNA fragments encoding the NC61a and 4575 epitopes fused with GST as well as GST (wild type) were amplified by PCR using the recombinant pGEX2T plasmids and non-recombinant pGEX2T, respectively, as templates. Primers used for PCR were:

NC61a-GST :

Forward ,5'CGCTCAG**CATATG**ATGTCCCCTATACTAGGTTATTG 3',

Reverse, 5'GAGGAAGAAGCTAATGATGGAACAGGAATAA**GGATCC**ATCTT 3'

4575-GST:

Forward, 5' CGCTCAG**CATATG**ATGTCCCCTATACYAGGTTATTG 3'

Reverse, 5'GGCCACAGGGGCCACCCGGCATCAGCTAG**GGATCC**ATCTG 3'

GST:

Forward, 5' ATTCG**CATATG**ATGTCCCCTATACTAGGTTATTG 3'

Reverse, 5' GCGACCATCCTCCAAAATAA**GGATCC**ATCT T 3'.

*Nde*I and *Bam*HI sites in the forward and reverse primers, respectively, are shown in bold, the stop codons in the reverse primers are underlined.

PCR products were analysed by agarose gel electrophoresis to confirm correct amplification and were digested with *Nde*I and *Bam*HI. In parallel, pET15b was digested with the same restriction enzymes. Digestion products were purified by preparative agarose gel electrophoresis as above. Digested amplification products were ligated into linearised pET15b with T4 ligase and electrocompetent *E. coli* was transformed with ligation reaction products as above. Transformants were screened by PCR and recombinant plasmids were confirmed by DNA sequence analyses.

2.2.2.4 Expression of recombinant proteins

Scale-up expression for recombinant protein purification was carried out in 1 L of LB supplemented with carbenicillin (50µg/ml) which was inoculated with 5ml of culture (LB/carbenicillin) grown for 16 hours from a single colony. Bacterial cultures were split in 2L-conical flasks (500 ml each) and grown at 37°C with shaking at 250 rpm to mid-exponential phase (OD_{600nm} = 0.7), when IPTG (1 mM final concentration) was added. Cells were harvested after 3 hours of -induction by centrifugation at 5000 x g for

20 min in a refrigerated centrifuge (4°C). Cell pellets were either processed immediately or were stored at -20 °C.

2.2.2.5 Purification of proteins

Cell pellets (from 1L cultures) were resuspended in 40 ml of 100mM Tris-HCl (pH 8.0) or PBS. An equal volume of Ballotini glass beads, size 12 (Jencons) was added and cells were lysed by homogenisation (3 bursts of 30 s duration at 6 m/s) using the Fast-prep 24 (MP Biomedicals, Santa Ana, California, USA). The glass beads were removed by centrifugation for 1 min at 1000 x g and the supernatant was transferred to centrifuge tubes (Nalgene, Rochester, NY, USA). Insoluble material was removed by centrifugation at 20,000 x g for 30 min at 4°C (Sorvall RC 5C PLUS) and recombinant proteins were purified from the supernatant as in the following sections.

2.2.2.5.1 Affinity chromatography using glutathione-Sepharose

Affinity chromatography was performed using 1 -2 ml Glutathione Sepharose 4B (GE Healthcare) in 10 cm or 5 cm x 1 cm diameter glass chromatography columns (Econo-Column; Biorad) at 4°C. Resin was washed with at least 10 column volumes (CVs) of PBS before loading of cleared lysates. Following loading of the lysates, columns were washed again with PBS (at least 10CVs until OD_{280nm} reached a base-line). Retarded components were eluted with 10mM glutathione in PBS and collected in 1 ml fractions. OD_{280nm} of each fraction was determined using the Nanodrop 2000. Aliquots of each fraction were analysed by SDS-PAGE.

2.2.2.5.2 Affinity chromatography using Ni^{2+}- resin

GST and GST-fusion proteins that were sub-cloned and expressed in pET15b express an additional $(His)_6$ affinity tag. Following purification using glutathione-Sepharose, these proteins were further purified by affinity chromatography with Ni^{2+} resin. Approximately 2 ml of chelating sepharose (GE Healthcare) was used for each recombinant protein. Columns were washed with 30 column volume (CV) of HPLC-grade water, then charged with Ni^{2+} by addition of 5 CV 50 mM NiSO4. Excess NiSO4 was removed by washing with 10 CV of HPLC-grade water. Resin was equilibrated with 10 CV of 5mM imidazole buffer (100mM Tris-HCl pH 8.0; 0.5 M NaCl; 5mM imidazole). GST and GST-fusion proteins eluted from glutathione-Sepharose columns were loaded directly. Columns were then washed with 5mM imidazole buffer to remove non-retarded components. Retarded fusion proteins were

eluted with 200mM imidazole, 0.5M NaCl in 100mM Tris-HCl (pH8.0). As before, fractions (1 ml) were collected, absorbance was measured and fractions were analysed by SDS-PAGE.

Mass spectroscopy analyses

Following SDS-PAGE electrophoresis of recombinant proteins, bands corresponding to GST, NC16a and 4575 were excised. Subsequently, at the Proteomics Facility, University of Bristol excised bands were digested with trypsin using the ProGest automated digestion unit (Perkin Elmer). Peptides were analysed using a 4700 MALDI-Tof/Tof mass spectrometer (Applied Biosystems, Foster City, CA) to give a peptide mass fingerprint and peptide sequence information.

2.2.2.6 ELISA

A 96-well plate was coated with GST, NC16a and 4575 at different concentrations; 2, 5 and 10 µg/ml in 100mM carbonate bicarbonate pH 9.6 incubated overnight at 4° C. It was then allowed to reach room temperature and washed with PBS/ T 20 3 times. The plate was blocked with PBS/ BSA/ Tween 20 which was the solution used as the assay diluent and was left at 37° C for one hr. After washing, 6 serum samples were added in duplicates in 6 doubling dilutions (1:100, 200, 400, 800, 1600 and 3200) and left incubating for 1 hr at 37° C. Subsequent to washing, the conjugate was added, an anti-human polyclonal IgG alkaline phosphatase linked antibody (1:1000) (A8542 SIGMA-ALDRICH) for 1 hr at 37° C. The reaction was stopped and the plate read as described above.

To reduce GST background reactivity, serum samples were incubated with GST lysate in order to reduce GST reactivity. Three sera samples were incubated with lysate at different concentrations; 2.5 and 10 fold of lysate with a 1:1 ratio of protease inhibitor (S8830 SIGMAFAST(TM) Protease Inhibitor Cocktail Tablets, EDTA-Free SIGMA-ALDRICH) prepared as per manufacturer's recommendations. In addition, different temperatures (4° C and room temperature), as well as incubation times of 1 hour and overnight were incorporated. Plates were blocked with PBS/ BSA/ Tween 20 for an hour at 37° C and washed 3x with PBS/ Tween 20 as previously described. Sera were incubated for 1 hr at 37° C and the anti-human polyclonal IgG conjugate was added (1:1000) (A8542 SIGMA-ALDRICH)

2.2.3 Surface Plasmon analyses of antibody binding to α6β4 integrin

The amine coupling procedure was used for immobilisation of α6β4 integrin. The surface of a CM5 sensor chip (GE Healthcare Lifesciences) was activated by injection of 0.2M EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) together with 0.05M NHS (N-hydroxysuccinimide) followed by injection of α6β4 integrin (5μg/ml in 10mM NaAcetate, pH4.0) over the surface of flowcell 2 (Fc2). No ligand was immobilised in Fc2 which served as a reference cell. Non-substituted groups on the dextran surface were blocked by injection of 1M ethanolamine-HCl, pH8.5. Approximately 2,000 RU were immobilised. Anti- α6 or anti- β4 antibodies (AF1350 and AF4060 R&D systems, respectively) were injected over both flow cells at a concentration of 1μM in HBS-P to confirm immobilisation of the integrin. Injection volume was 40μl, buffer was HBS-P and flow rate was 10μl/min. The sensorchip surface was regenerated by injection of 100mM diethylamine. The same conditions were used to investigate binding of sera (diluted in HBS-P) to the immobilised integrin.

2.3 Statistical analyses

Statistical analyses were undertaken using the GraphPad Prism 5.02. One-way analysis of variance (ANOVA) was used to compare the mean of the different subgroups tested i.e.; MMP, PV, HC and DC. Positivity for either MMP or PV for any of the variables tested (IgG serum and saliva, IgA serum and saliva or IgA and SIgA parotid) was defined as above the mean +2SD of the healthy controls. Bonferroni and Newman-Keuls corrections for all post-anova comparison tests were used. Associations between antibody titres and disease severity and clinicopathological associations were conducted by Chi square and Spearman Rank correlation for non-parametric data. Sequential results' analyses were conducted by taking the difference between the first and final time point in sample collection and performing Spearman correlation between the change in antibody titre and the oral disease severity score for MMP analysis. Logistic regression was used for the analysis of the sequential data in PV. The mean of the coefficient of variance was used to establish reproducibility of the ELISA tests based on a positive sample used repeatedly on the plates. Intra-and inter-plate variations were both calculated.

3 Results MMP Study 1 (a&b)

Serum and salivary antibodies to BP180-NC16a in patients with mucous membrane pemphigoid

(a)- Serum and salivary antibodies to BP180-NC16a in relation to clinical phenotype and disease activity (Cross-sectional)

(b)- Serum and salivary antibodies to BP18-NC16a in relation to therapeutic response (Longitudinal)

3 Serum and salivary antibodies to BP180-NC16a in patients with mucous membrane pemphigoid (MMP study 1 a&b)

3.1 Introduction

The main target antigens lie within the hemidesmosome; these include bullous pemphigoid antigen 2 (BP180), laminin 332, alpha 6 beta 4 (α6β4) integrin and less commonly collagen VII (Schmidt and Zillikens, 2013). There is still much controversy regarding specific target antigens and clinical phenotype. The beta 4 integrin has been implicated in ocular MMP (Tyagi et al., 1996) while the alpha 6 integrin has been mentioned with oral MMP (Rashid et al., 2006b, Bhol et al., 2001). However, other studies state that BP180 is the main antigen targeted in oral or multisite MMP (Schmidt et al., 2001b, Carrozzo et al., 2004, Calabresi et al., 2007).

Indirect immunofluorescence (IIF) on salt-split skin (SSS) can be used to monitor serum titres of IgG and IgA for the assessment of disease activity whereas enzyme-linked immunosorbent assays (ELISA) are not yet used routinely for monitoring disease activity. Dual serum antibodies (IgG and IgA) have been shown to be associated with a more severe disease (Setterfield J. et al., 1998).

Testing serum for antibodies to basement membrane zone (BMZ) antigens by ELISA to diagnose MMP has been well documented (Calabresi et al., 2007, Sciubba, 2011, Chen et al., 1997, Bekou et al., 2005). Specific target antigens, such as the NC16a domain of BP180, BP230 or laminin 332 have been used for serum testing and are documented (Bekou et al., 2005, Wieland C. N. et al., 2010). In contrast, the use of **saliva** as a test fluid has been reported only once to our knowledge and reported negative results (Andreadis D, 2006).

The aims of this study were:

1) To identify the whether serum and salivary IgG and/or IgA autoantibodies to BP180-NC16a were present in our cohort of MMP
2) To utilize serum and salivary biomarkers in the analysis of disease activity and therapeutic responses
3) To identify whether there was an association with carefully defined clinical subgroups

In this study both whole and parotid saliva were tested for the presence of antibodies to BP180-NC16a. Saliva is a potentially valuable substrate as it reflects many components of the systemic circulation such as antibodies and cytokines (Mandel, 1990). Additionally parotid saliva was tested in order to differentiate between IgA in whole saliva as a serum transudate and/ or as mucosally derived antibody (i.e. whether it was monomeric or dimeric SIgA). Antibodies in either fluid were related to disease activity to determine if a relationship between antibody titre and disease severity could be established. Sequential samples were also analysed in order to deduce whether a relationship existed between changes in clinical severity (therapeutic responses) and any changes in antibody titre.

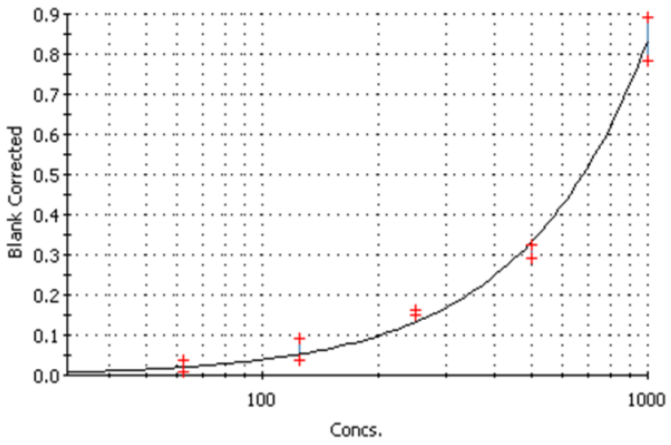
Furthermore, in an attempt to ascribe a relationship between specific clinical phenotypes and definite target antigens, the cohort was sub-grouped into pure oral, pure ocular and multisite MMP. Recombinant protein was expressed and purified to optimize ELISA testing for the C-terminal domain BP180-4575 (see methods).

3.2 A) Serum and salivary antibodies to BP180-NC16a in relation to clinical phenotype and disease activity (Cross-sectional study 1a)

3.2.1 Serum and whole saliva

In this study (Study 1 a), serum and whole saliva samples from MMP patients (n=78), healthy controls (n=50) and disease controls (PV n=6, LP n=16) were tested for antibodies against BP180-NC16a by ELISA. The standard curves against which all serum and whole saliva samples were tested are shown in (Fig 3-1 a-b).

a)



b)

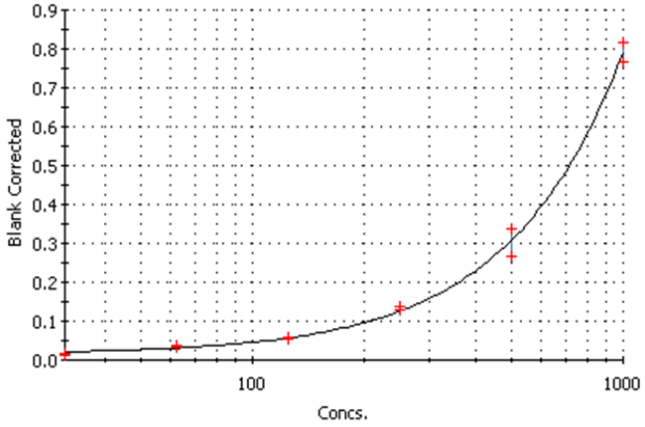
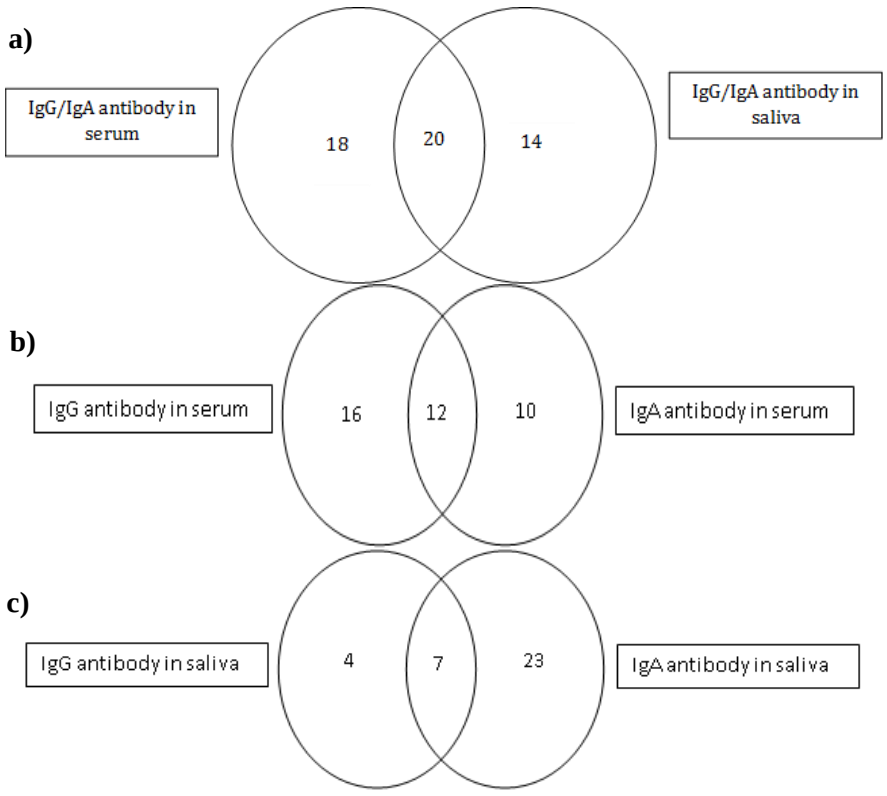


Figure 3-1 Standard curve for serum and whole saliva IgG/IgA antibody testing against BP180-NC16a by ELISA a) Standard curve for serum and whole saliva IgG antibody testing against BP180-NC16a by ELISA. b) Standard curve for serum and whole saliva IgA antibody testing against BP180-NC16a by ELISA. Both figures show a Y-axis which represents blank-corrected optical density values and an x-axis representing arbitrary units (0-1000) (100,000 by multiplying by dilution factor 100). The standard curve was constructed using 6 doubling dilutions of a range of pooled antibody positive serum samples. Serum and whole saliva IgG/IgA antibody titres were read in units by calculating the mean of values of four doubling dilutions falling on the reference curve

A summary of patients' demographics, clinical data, immunofluorescence and ELISA results are shown in Appendix 3. The mean titres for both IgG antibodies (19062 ± 34710) and IgA antibodies (13514±16639) in serum and saliva (740.9±2243)/ (1701±2273) from MMP patients, were significantly different from those of the controls (both healthy and disease) in all tested parameters using one-way ANOVA ($p<0.0001$). Values greater than the mean +2 SD of the healthy controls were considered positive. Healthy and disease control samples were all negative using these criteria. The negative predictive values were 65% for IgG serum, 59% for IgA serum, 62% for IgA saliva and 56% for IgG saliva. Serum IgG antibodies against the NC16a domain of BP180 were detected in 28/78 (36%) of MMP patients (Fig 3-3). Serum IgA antibodies were found

in 22/78 (28%) (Fig 3-4). Combined serum IgG and/or IgA antibodies were positive in 38/78 (48%) patients (Fig 3-2 b).

In whole saliva, IgA antibodies were detected in 30/78 (38%) of MMP patients (Fig 3-5). IgG antibodies were detected in 11/78 (14%) of MMP whole saliva samples (Fig 3-6), all of whom were also positive in serum for IgG antibodies and all eleven had active oral disease with oral scores indicating inflammation (The Oral Disease Severity Score ranged between 5-35 in the positive samples). Combined whole saliva IgG and/or IgA antibodies were positive in 34/78 (44%) (Fig 3-2 c). Results are listed in Table 3-1. IgG antibody in serum and saliva to BP180-NC16a was detected in 28/78 (36%) MMP patients (Fig 3-2 d). IgA antibody in serum and/or IgA antibody in saliva to BP180-NC16a were detected in 41/78 (52%) patients (Fig 3-2 e). Overall, 52/78 (67%) were positive for IgG and/or IgA antibodies to BP180-NC16a in serum and saliva or both (Fig 3-2 a).



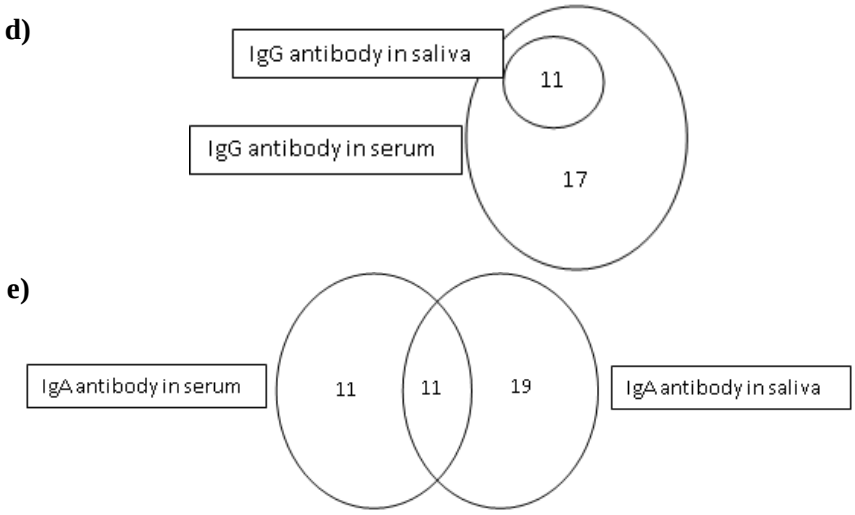


Figure 3-2 Venn diagram showing relation between antibodies (IgG/IgA) to BP180-NC16a detected in serum and whole saliva

a) IgG and/or IgA antibody in serum and saliva (whole and/or parotid) was detected in 52/78 (67%) b) IgG and/or IgA antibody in serum detected in 38/78 (48%) MMP patients. c) IgA antibody in saliva and/or IgG antibody in saliva was detected in 34/78 (44%) MMP patients d) IgG antibody in serum and saliva was detected in 28/78 (36%) MMP patients e) IgA antibody in serum and/or saliva was detected in 41/78 (52%) MMP patients

Table 3-1 Study 1a ELISA results for IgG and IgA antibodies against BP180-NC16a in serum and whole saliva.

Study 1 (a) MMP (n=78)	IgG Serum	IgG Whole Saliva	IgA Serum	IgA Whole Saliva
ELISA results against BP180-NC16a	28/78 (36%) Combined IgG and IgA in serum 38/78 (48%)*	11/78 (14%) Combined IgG in serum and whole saliva 28/78 (36%)	22/78 (28%) Combined IgA in serum and whole saliva 41/78 (52%)*	30/78 (38%) Combined IgG and IgA in whole saliva 34/78 (44%)*

* combined: total of those expressing either IgG or IgA antibodies or both

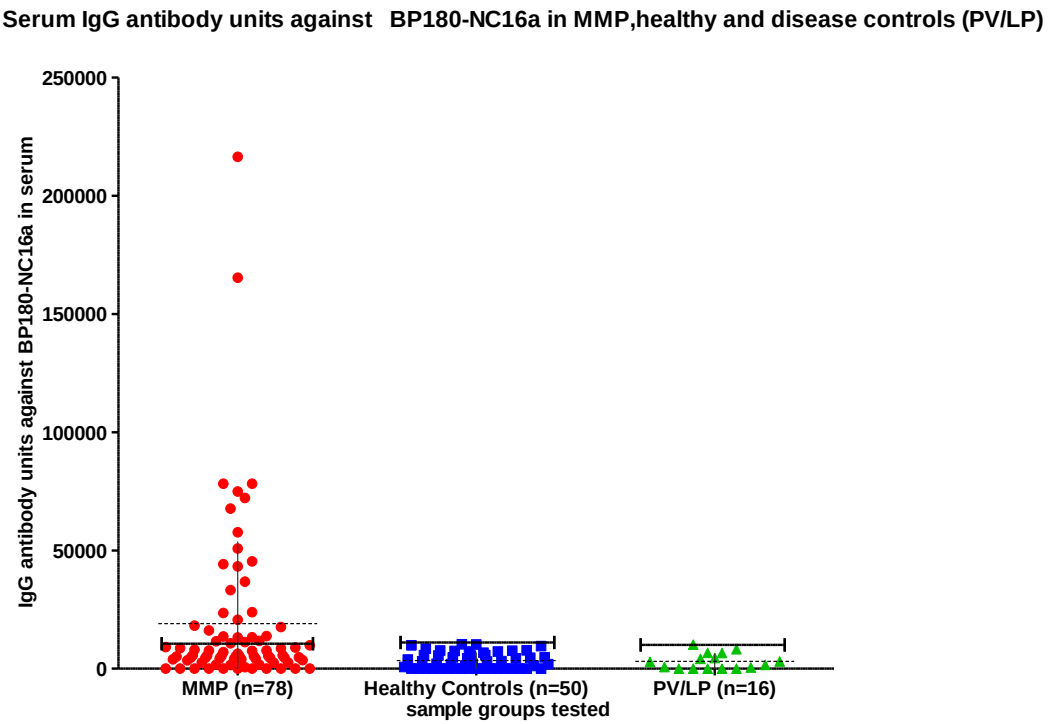


Figure 3-3 Serum IgG antibodies against BP180-NC16a in MMP paients healthy and disease controls using ELISA (Mean +/- SD) The Y-axis represents the antibody titre in units while the X-axis shows the sample groups tested. Horizontal I bar indicates cut-off level (mean + 2SD of healthy controls). Controls were negative in all. MMP positive in 28/78 (36%)

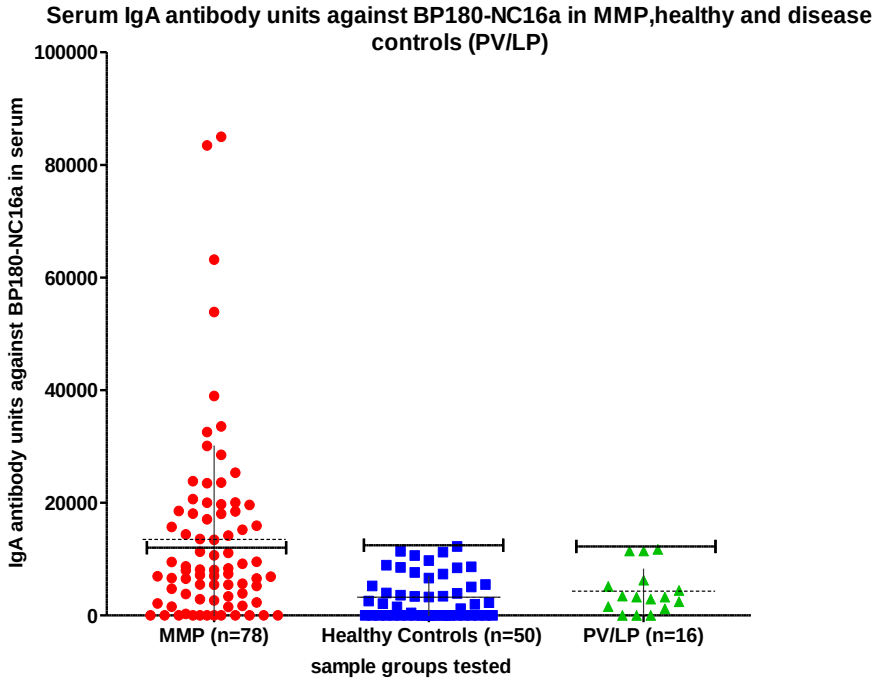


Figure 3-4 Serum IgA antibodies to BP180-NC16a ELISA in MMP patients, Healthy and Disease controls using ELISA (Mean +/- SD) The Y-axis represents the antibody titre in units while the X-axis shows the sample groups tested. Horizontal I bar indicates cut-off level (mean + 2 SD of the HC). Controls were negative in all. Serum IgA antibody units against BP180-NC16a in MMP were positive in 22/78 (28%)

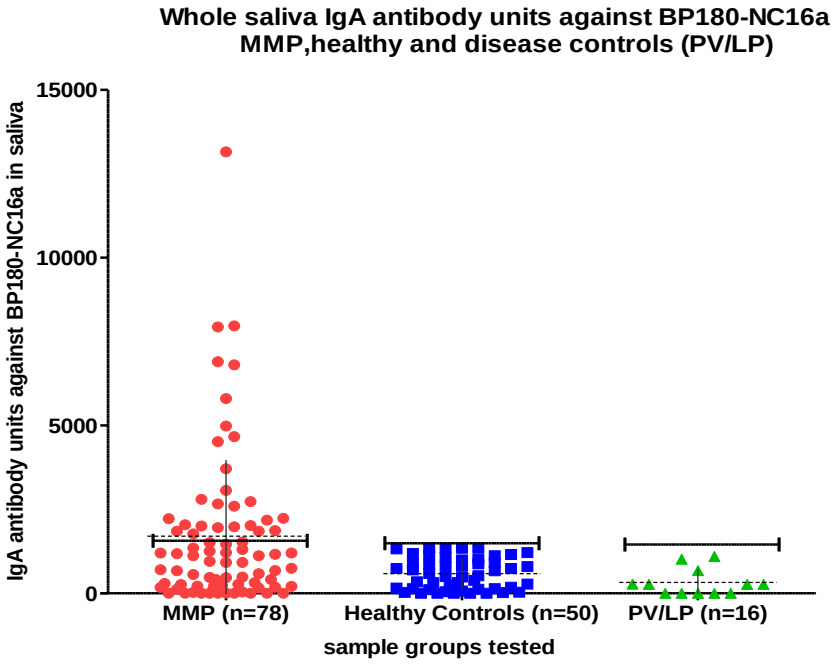


Figure 3-5 IgA antibodies to BP180-NC16a ELISA in whole saliva in MMP patients, Healthy and Disease controls using ELISA (Mean +/- SD) The Y-axis represents the antibody titre in units while the X-axis shows the sample groups tested. Horizontal I bar indicates cut-off level (mean + 2 SD of the HC). Controls were negative in all. Whole saliva IgA antibody units against BP180-NC16a in MMP were positive in 30/78 (38%)

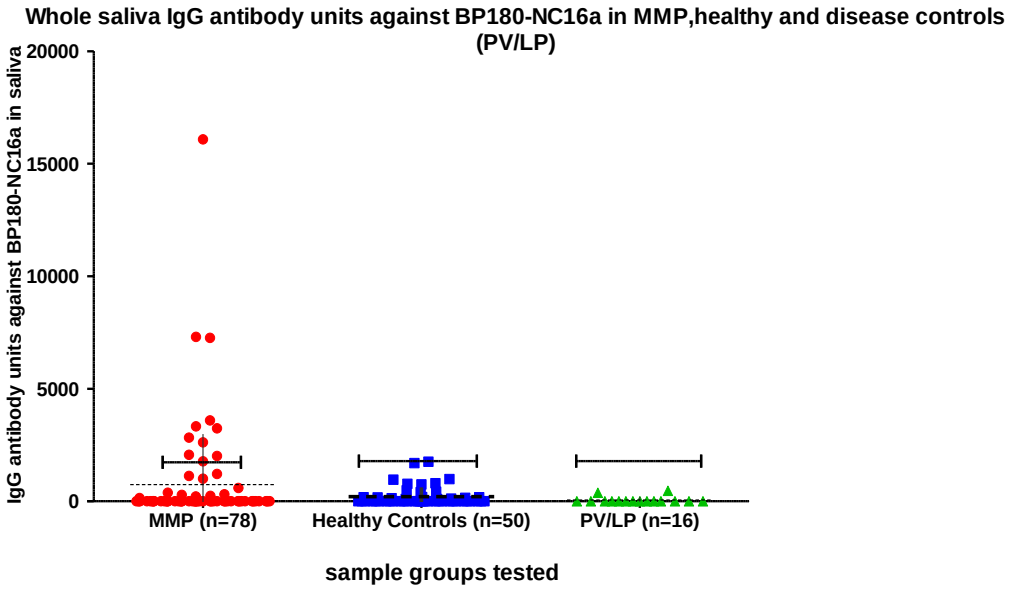


Figure 3-6 IgG antibodies to BP180-NC16a ELISA in whole saliva in MMP patients, Healthy and Disease controls using ELISA (Mean +/- SD) The Y-axis represents the antibody titre in units while the X-axis shows the sample groups tested. Horizontal I bar indicates cut-off level (mean + 2 SD of the HC). Controls were negative in all. Whole saliva IgG antibody units against BP 180-NC16a in MMP were positive in 11/78 (14%)

Additional immunoblotting results from the collaborators in Germany (Schmidt/Zillikens) on MMP samples from this study

Immunoblotting studies to detect serum IgG or IgA antibodies to BP180-4575 (C-terminal domain) and Laminin 332, were performed and provided by our collaborators. They tested serum from 39 patients we provided from this study for IgG and IgA antibodies to BP180-4575 domain as well as IgG antibody to Laminin 332 by western blot. Serum IgG antibodies to BP180-4575 were positive in 7/39 (18%) patients. Eight of 39 (21%) were positive for serum IgA antibodies to BP180-4575. Finally, 6/39 (15%) were positive for serum IgG antibodies to laminin 332.

The results are summarised in (Table 3-2). A comparison of results for antibodies to BP180-4575 from our collaborators and antibodies to BP18-NC16a in our ELISA results showed that all samples tested were positive for both except 4 samples which were positive for IgG and/or IgA against BP180-4575 but were negative with our ELISA tests (BP180-NC16a and/or α6β4 integrin). These 4 negative samples were categorised as 3 of the pure oral subgroup and 1 of the pure ocular. Similarly, three of the 6 samples positive to laminin 332 were positive with our ELISA tests while 3 were negative with our ELISA tests (BP180-NC16a and/or α6β4 integrin). Of these 3 negative samples, two were multisite disease subgroup and 1 was pure oral.

Table 3-2 Summary of MMP serum samples tested against BP180-NC16a and 4575, α6β4, and Laminin 332 using ELISA and western blot

Antigen	BP180-NC16a	BP180-NC16a	*BP180-4575	*BP180-4575	α6β4	*Laminin 332
Testing method	ELISA	ELISA	Western blot	Western blot	ELISA	Western blot
Immunoglobulin detected	IgG	IgA	IgG	IgA	IgG	IgG
Positivity	28/78 (36%)	22/78 (28%)	7/39 (18%)	8/39 (21%)	36/100 (36%)	6/39 (15%)

*Indicates collaborators' results

Relationship between serum and saliva antibodies to NC16a (data from study 1 a) and clinical phenotype

When data were analysed for an association with clinical phenotype, there was a positive association between the presence of serum IgG antibodies to BP180-NC16a and multisite disease lesions ($X^2 = 11.9$ p =0.003). A significant relation was also found between the presence of IgA antibodies against BP180-NC16a in whole saliva and the presence of both oral and ocular disease ($X^2 = 6.23$ p =0.016). There was no apparent association with other clinical subgroups (pure oral or pure ocular) or disease severity (Table 3-3).

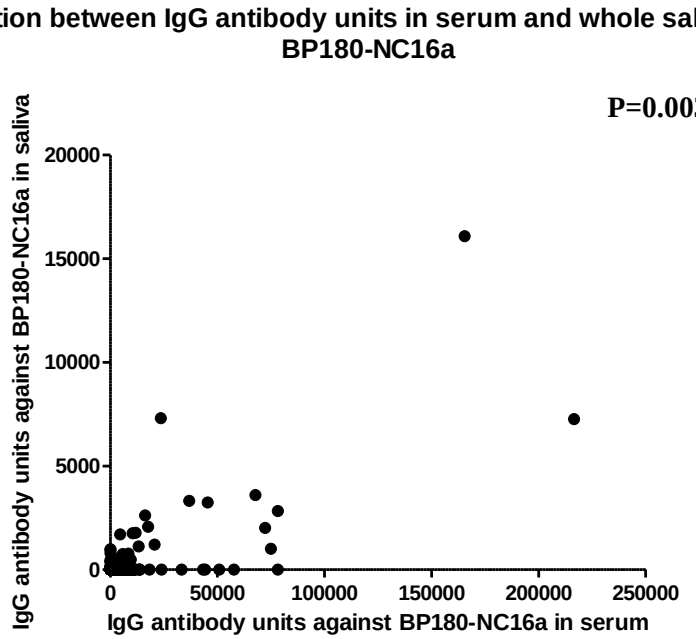
There was a positive correlation between IgG antibodies to BP180-NC16a in serum and saliva (Spearman r =0.244 p =0.003) (Fig 3-7 a). A positive association was also found between IgG and IgA antibodies to BP180-NC16a in serum (Spearman r =0.303 p =0.0002) (Fig 3-7 b). No statistically significant correlation was found between IgA antibodies to BP180-NC16a in serum and saliva. No statistically significant correlation was found between IgA antibodies to BP180-NC16a in whole saliva and the oral disease severity score (Spearman r=-0.099 p=0.385). However, a positive association was found between IgG antibody to BP180-NC16a secretory rate in whole saliva and the oral disease severity score (Spearman r=0.225 p=0.048) (Fig 3-8).

Table 3-3 Statistical analysis of data according to clinical subgroups. Antibodies to BP180-NC16a (IgG and IgA) in serum and whole saliva and direct and indirect immunofluorescence results in relation to clinical phenotypes of pure oral, pure ocular, multisite disease.

	Pure Oral (n=23)	**Pure Ocular (n=16)**	**Multisite Disease (n=50)**	**Overall**	**Chi square p value**
IgG Serum	8/23 (35%)	0/16	20/50 (50%)	28/78 (36%)	11.9 p=0.003 **
IgA Serum	3/23 (13%)	5/16 (33%)	14/50 (35%)	22/78 (28%)	3.7 p=0.16
IgG Whole saliva	3/23 (13%)	0/16 (0%)	8/50 (20%)	11/78 (14%)	3.6 p=0.16
IgA Whole saliva	10/23 (44%)	7/16 (47%)	13/50 (33%)	30/78 (38%)	1.3 p=0.53 (**combined oral and/or ocular 6.23 p=0.016*)**
DIF	21/22 (96%)	5/16 (39%)	31/39 (80%)	57/74 (77%)	15.3 p=0.000 **
IIF	7/18 (39%)	0/16	10/21 (48%)	23/65 (35%)	1.1 p=0.59

DIF (Direct immunofluorescence), IIF (Indirect immunofluorescence), * significant, ** highly significant

a)



b)

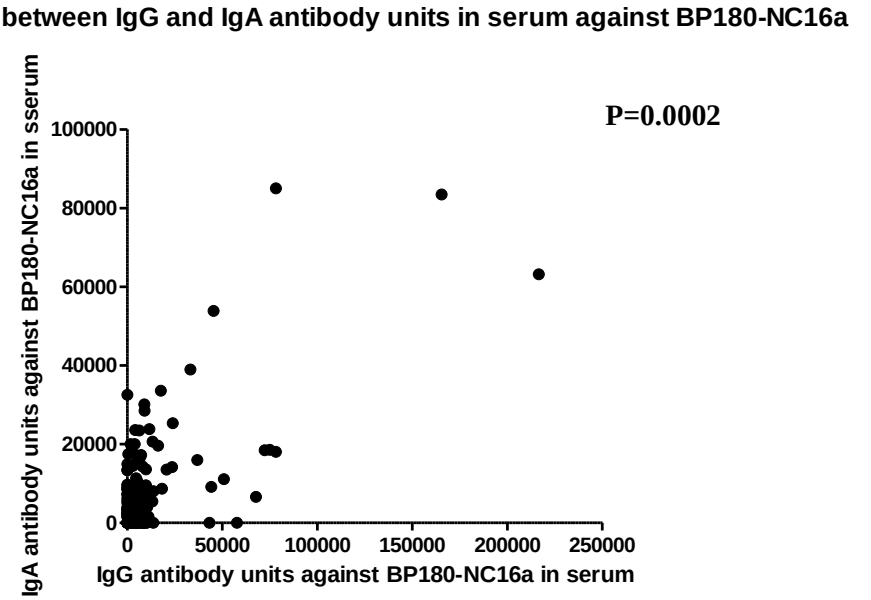


Figure 3-7 Correlations (Spearman rank correlation for non-parametric data) between: a) IgG antibodies to BP180-NC16a in serum and whole saliva (Spearman r =0.244 p=0.003) and b) IgG and IgA antibodies to BP180-NC16a in serum (Spearman r =0.303 p =0.0002)

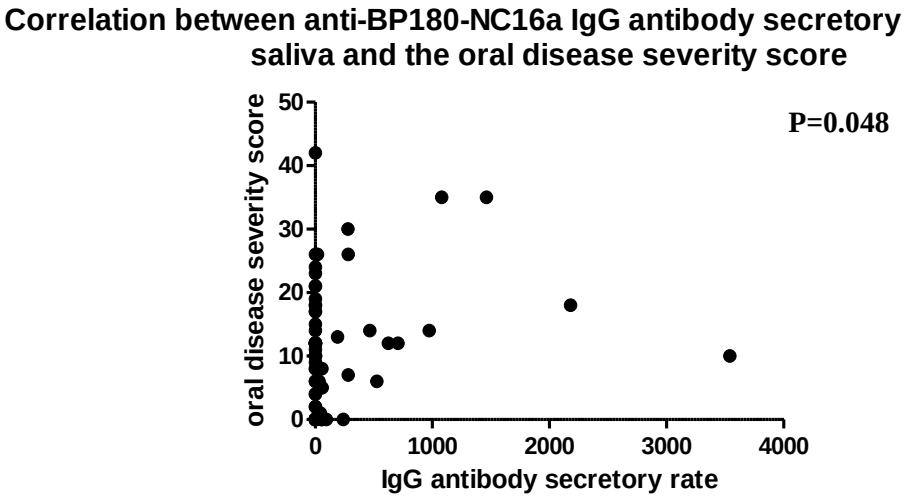


Figure 3-8 Spearman rank correlations of anti-BP180-NC16a antibody secretion rate in whole saliva and the oral disease severity score Correlation of anti-BP10-NC16a IgG antibody secretion rate in whole saliva and the oral disease severity score (Spearman r=0.225 p=0.048)

Sensitivity of assays

The sensitivity of detecting IgG antibody against BP180-NC16a in whole saliva was tested by the spiked ELISA described (see chapter 2). The standard was spiked to test whether IgA or salivary components such as mucins were inhibiting IgG detection. The results showed that the technique was sensitive as there was no difference between the standards (all three lines representing the various conditions overlap) (Fig 3-9).

Antibodies in relation to clinical phenotype

The MMP samples tested in both cross-sectional and longitudinal studies (n=91) were further analysed by subgrouping the patients based on clinical phenotype; pure oral (n=25), pure ocular (n=16) and multisite (n=50) (Fig 3-10 and 3-11) (results summarized in Table 3-4).

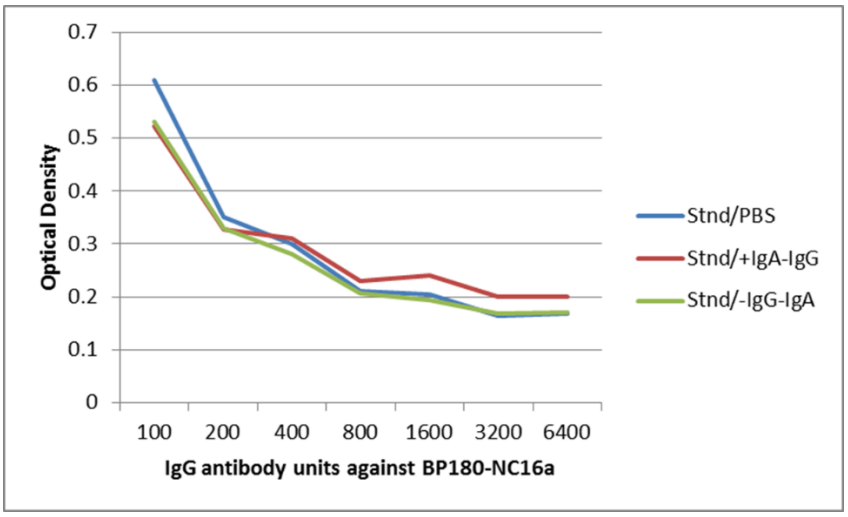


Figure 3-9 Three spiked standards were used to establish the sensitivity of IgG antibody detection against BP180-NC16a using the commercially available pre-coated ELISA plates (blue on graph) standard spiked with PBS, (red on graph) standard spiked with a whole saliva sample positive for IgA antibody against BP180-NC16a but negative IgG antibody, (green on graph) standard was spiked with a whole saliva sample negative for both IgG and IgA antibodies against BP180-NC16a. Y-axis in the OD and X-axis represents units of IgG antibody to BP180-NC16a

Serum antibodies in relation to clinical phenotype

The pure ocular MMP subgroup was found to be negative for IgG antibody against BP180-NC16a (0/16). In the pure oral subgroup, 36% (9/25) were positive for serum IgG antibody against BP180-NC16a, while in the multisite group 46% (23/50) were positive for IgG antibody against BP180-NC16a. With regard to IgA antibody in serum against BP180-NC16a, 38% (6/16) were positive in the pure ocular group (the highest percentage), 16% (4/25) were positive in the pure oral group and 28% (14/50) were positive in the multisite group. Thus, in the pure ocular group, serum antibodies to NC16a were of the IgA isotype and not IgG.

Salivary antibodies in relation to clinical phenotype

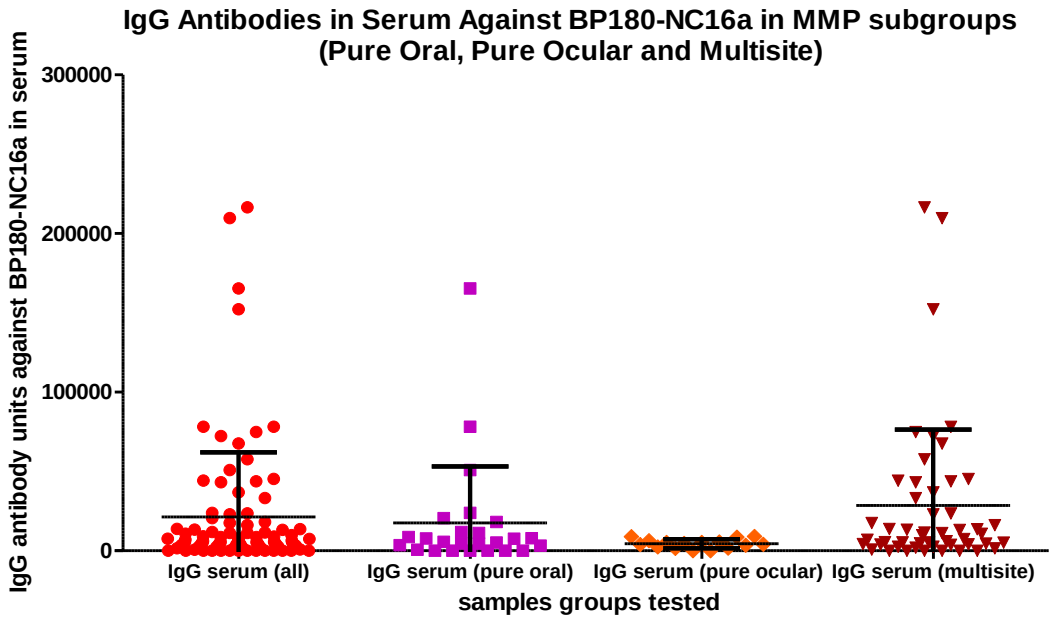
In whole saliva, IgG antibody against BP180-NC16a was positive in 16% (4/25) in the pure oral group and 18% (9/50) in the multisite groups with none being positive in the pure ocular subgroup. IgA antibody in whole saliva against BP180-NC16a was positive in 44% (7/16) in the pure ocular subgroup in 40% (10/25) of the pure oral subgroup and in 30% (15/50) of the multisite group (Table 3-4). Thus overall, a higher percentage of patients showed salivary IgA antibodies to NC16a than serum IgA antibodies (32 of 91 (35%) versus 24 of 91 (26%).

Table 3-4 Results of IgG and IgA antibodies in serum and whole saliva against BP180-NC16a ELISA by clinical phenotype distribution.

Study by clinical phenotype MMP (n=91) against BP180-NC16a	Pure Oral (n=25)	Pure Ocular (n=16)	Multi-site (n=50)
IgG Serum	9/25 (36%)	0	23/50 (46%)*
IgG Whole Saliva	4/25 (16%)	0	9/50 (18%)*
IgA Serum	4/25 (16%)	6/16 (38%)*	14/50 (28%)
IgA Whole Saliva	10/25 (40%)	7/16 (44%)*	15/50 (30%)

* represents the highest percentage value of autoantibody detected

a)



b)

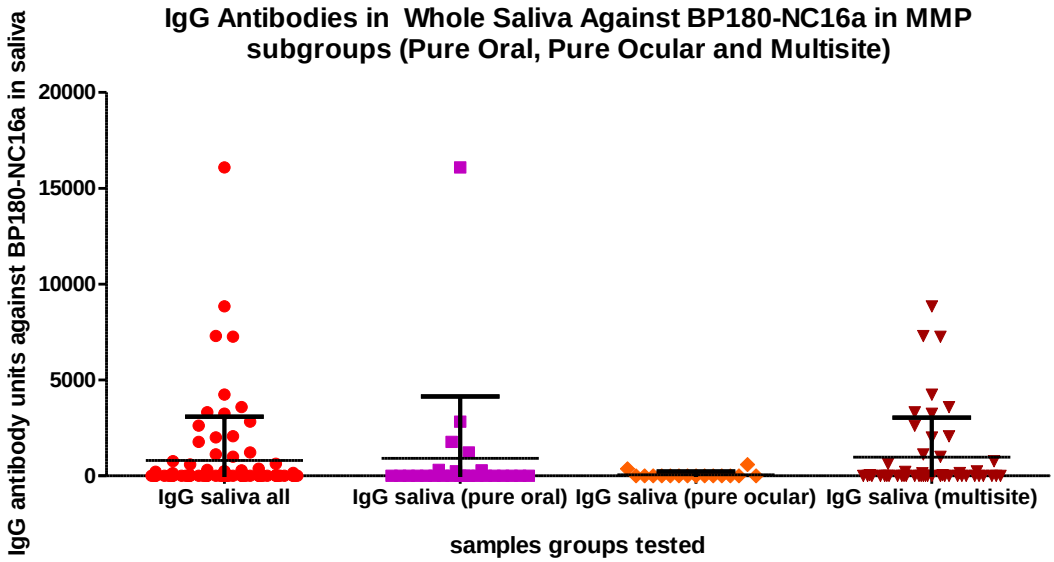
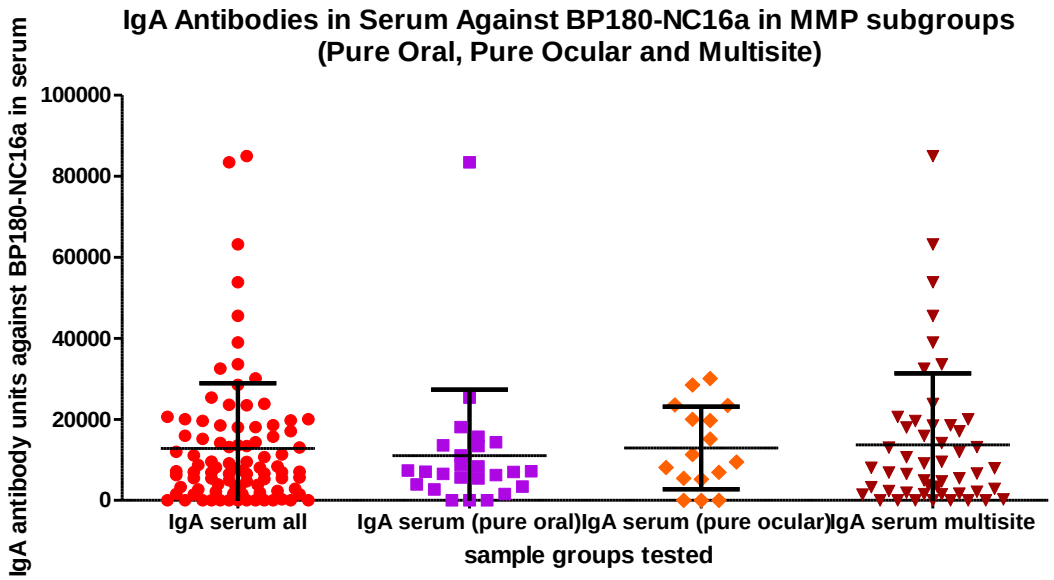


Figure 3-10 MMP patients subgrouped according to clinical phenotype and tested for IgG antibodies against BP180-NC16a in serum and whole saliva (Mean +/- SD)
Subgroups include MMP cohort (study a+b) (n=91), pure oral (n=25), pure ocular (n=16) and multisite (n=50). The Y-axis represents the units of each specific antibody in the medium tested. The X-axis corresponds to the sample groups tested. Positivity was established at the mean + 2SD of the HC group (see methods). Controls were negative (See Fig 3.2-3.5). Comparisons were drawn between the MMP subgroups only. a) IgG antibody units in serum against BP180-NC16a 32/91 (35%) positive in MMP(all), pure oral 9/25 (36%), pure ocular (0/16) and multisite group 23/50 (46%) b) IgG antibody units in whole saliva against BP180-NC16a 13/91 (14%) positive in MMP (all), pure oral 4/25 (16%), pure ocular (0/16) and the multisite group 9/50 (18%)

a)



b)

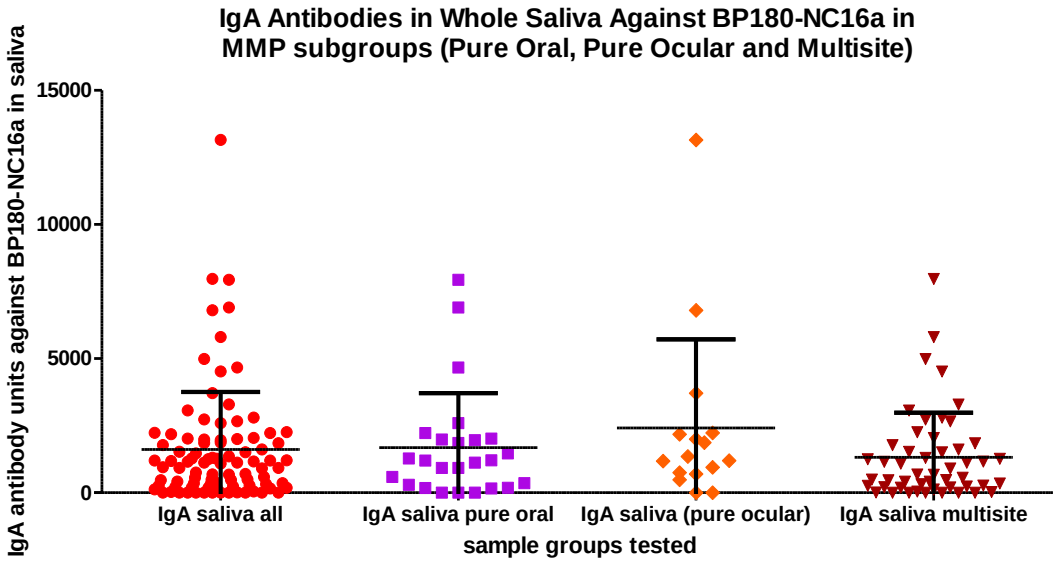


Figure 3-11 MMP patients subgrouped according to clinical phenotype and tested for IgA antibodies against BP180-NC16a in serum and whole saliva (Mean +/-SD)
Subgroups include MMP cohort (study a+b) (n=91), pure oral (n=25), pure ocular (n=16) and multisite (n=50). The Y-axis represents the units of each specific antibody in the medium tested. The X-axis corresponds to the sample groups tested. Positivity was established at the mean + 2SD of the HC group (see methods). Controls were negative (see Fig. 3.2-3.5) Comparisons were drawn between the MMP subgroups only. a) IgA antibody units in serum against BP180-NC16a 24/91 (26%) positive in MMP (all), pure oral 4/25 (16%), pure ocular showed 6/16 positivity (38%) and the multisite group 14/50 (28%). b) IgA antibody units in whole saliva against BP180-NC16a were positive 33/91 (36%) of MMP (all), pure oral 10/25 (40%), pure ocular showed 7/16 positivity (44%) and the multisite group 15/50 (30%)

b) Relationship between antibodies to BP180-NC16a and Disease severity

There was a positive relation between the units of IgG antibody in whole saliva against BP180-NC16a and the oral disease severity score in the multisite subgroup (Spearman r =0.323 p =0.022) (Fig 3-12), and also a positive correlation between the units of IgG antibody in serum and in saliva against BP180-NC16a and the number of sites involved (Spearman r =0.335 p =0.017 and Spearman r =0.329 p =0.019, respectively) (Fig 3-13 a-b). A positive association was also found between IgG antibody units against BP180-NC16a in serum and saliva in both the pure oral and multisite groups (Spearman r =0.483 p =0.015 and Spearman r =0.569 p <0.0001, respectively) (Fig 3-14 a-b). Similarly, a positive relation was found between IgG and IgA antibody units against BP180-NC16a in serum in both the pure oral and the multisite groups (Spearman r =0.495 p =0.012 and Spearman r =0.454 p =0.0009, respectively) (Fig 3-15 a-b). Correlations are summarized in Table 3-5

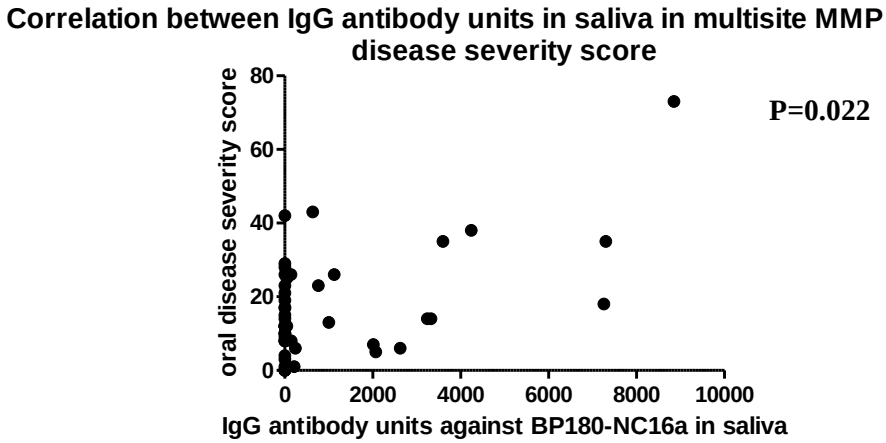
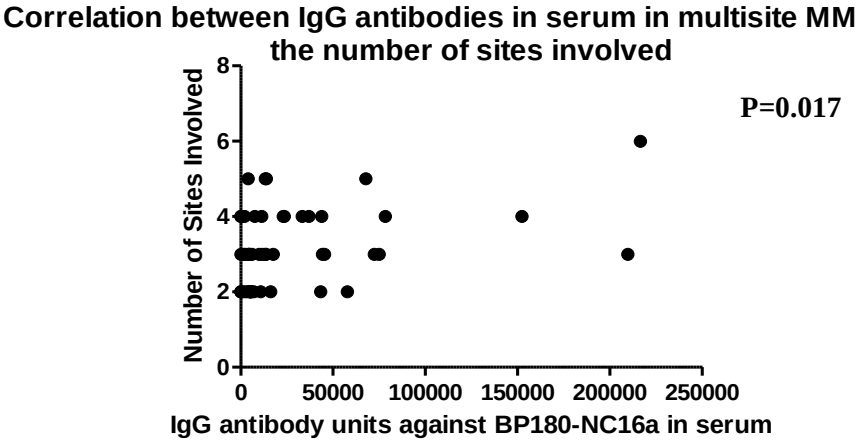


Figure 3-12 The correlation between IgG antibodies in whole saliva against BP180-NC16a in the multisite subgroup of MMP and the oral disease severity scores (Spearman r =0.323 p =0.022) (Details of oral disease severity scores in methods)

a)



b)

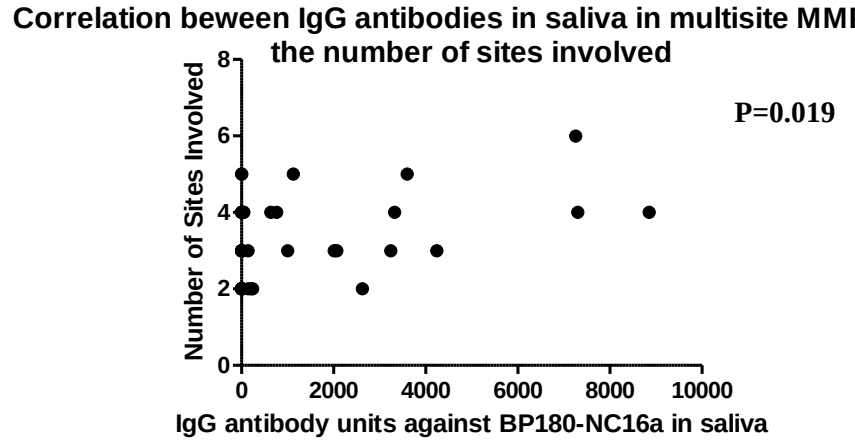
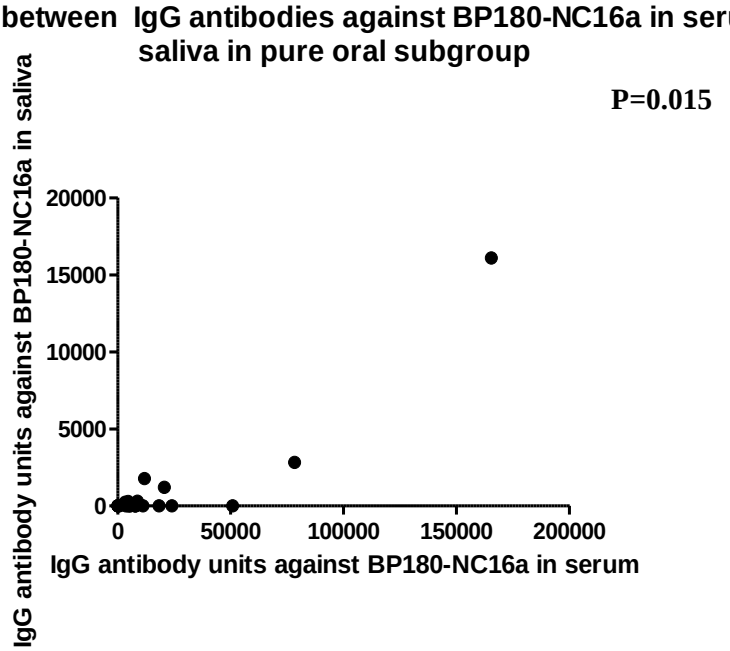


Figure 3-13 The correlation between IgG antibodies to BP180-NC16a in both serum and whole saliva and number of sites involved in the multisite subgroup a) The correlation between IgG antibodies to BP180-NC16a in *serum* and number of sites involved in the multisite subgroup (Spearman r =0.335 p =0.017). b) The correlation between IgG antibodies to BP180-NC16a in *whole saliva* and number of sites involved in the multisite subgroup (Spearman r =0.329 p =0.019)

a)



b)

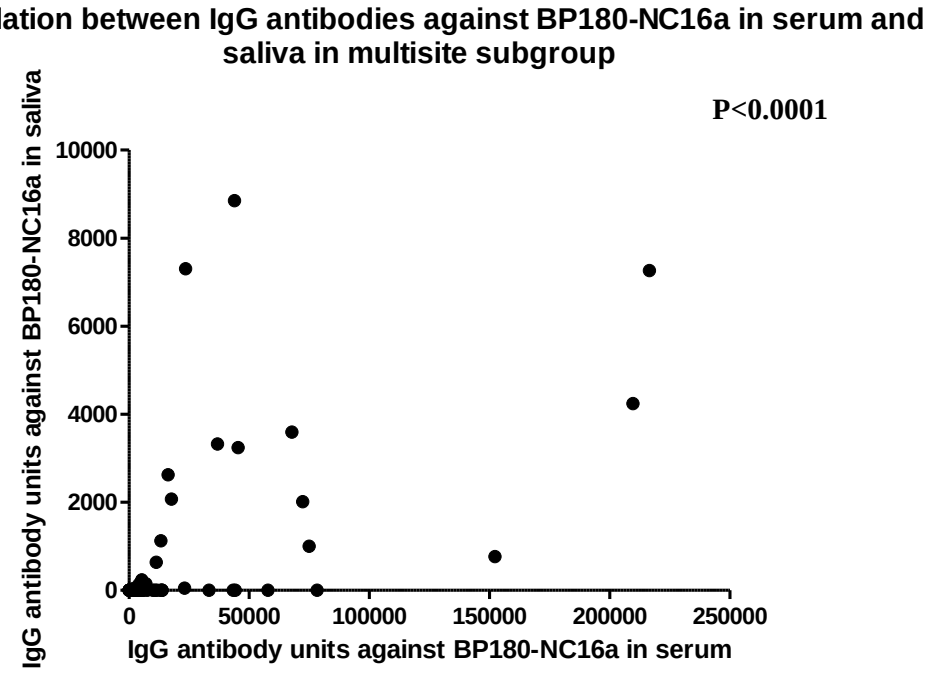
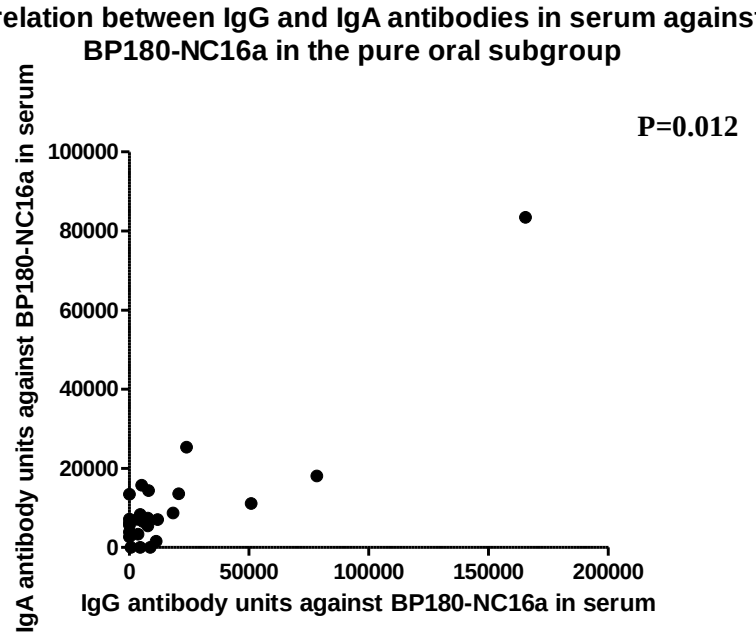


Figure 3-14 The correlation between IgG antibodies toBP180-NC16a in both serum and whole saliva in the pure oral and multisite subgroups a) The correlation between IgG antibodies to BP180-NC16a in serum and saliva in the ***pure oral*** subgroup (Spearman r = 0.483 p =0.015). b) The correlation between IgG antibodies to BP180-NC16a in serum and saliva in the ***multisite subgroup*** (Spearman r =0.569 p <0.0001)

a)



b)

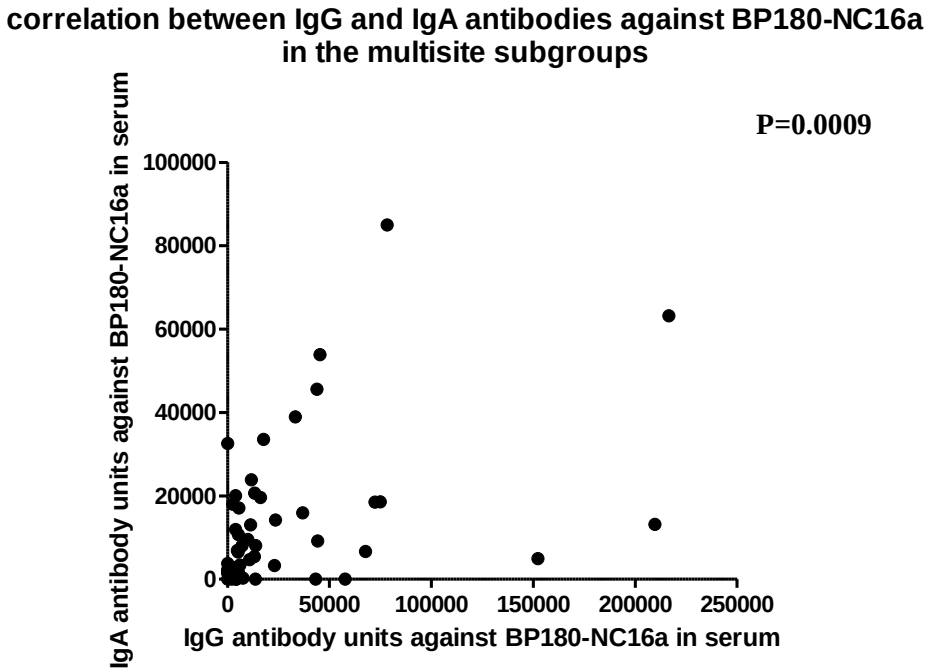


Figure 3-15 The correlation between IgG and IgA antibodies to BP180-NC16a in serum in the pure oral and multisite subgroups a) The correlation between IgG and IgA antibodies to BP180-NC16a in serum in the *pure oral* subgroup (Spearman r =0.495 p =0.012). b) The correlation between IgG and IgA antibodies to BP180-NC16a in serum in the *multisite* subgroup (Spearman r =0.454 p =0.0009)

Table 3-5 Summary of correlations between serum and salivary antibodies to BP180-NC16a and the disease severity in different clinical phenotypes

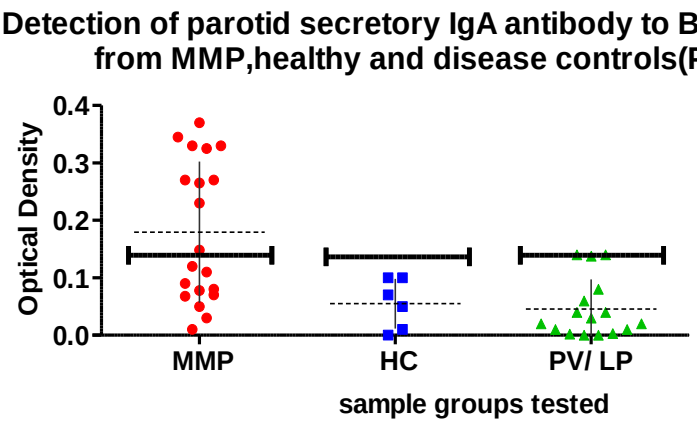
Variables	Clinical phenotype	P Value
IgG antibodies to BP180-NC16a in serum and whole saliva	Pure oral	Spearman r=0.4826 p= 0.015
IgG antibodies to BP180-NC16a in serum and whole saliva	Multi-site disease	Spearman r=0.5688 p<0.0001
IgG and IgA antibodies to BP180-NC16a in serum	Pure oral	Spearman r= 0.4953 p= 0.012
IgG and IgA antibodies to BP180-NC16a in serum	Multi-site disease	Spearman r= 0.4535 p= 0.0009
IgG antibodies to BP180-NC16a in serum and number of sites involved in multi-site disease patients	Multi-site disease	Spearman r= 0.3351 p= 0.017
IgG antibodies to BP180-NC16a in whole saliva and number of sites involved in multi-site disease patients	Multi-site disease	Spearman r= 3286 p= 0.019
IgG antibodies to BP180-NC16a in whole saliva and the oral disease severity score	Multi-site disease	Spearman r=0.3227 p=0.022

3.2.2 Detection of secretory IgA antibodies to NC16a in Parotid Saliva

Antibodies in whole saliva may be derived from serum as transudate, locally produced antibodies within the mucosal system or a combination of both. Antibodies in parotid are predominantly secretory IgA. Thus whole and parotid saliva were evaluated for both IgA and SIgA antibodies to BP180-NC16a. Antibodies against BP180-NC16a were detected in parotid saliva in 9/20 (45%) patients and were positive for secretory component in all nine (Fig 3-16 a). In addition, the antibodies detected by anti-IgA and by anti-secretory component were strongly correlated (Spearman r=0.9141) (Fig 3-16 b). Amongst the 9 positive for SIgA antibodies in parotid samples, all were also positive for IgA antibodies in whole saliva (annotated as * in Appendix 3) but 7/9 were negative in serum. Conversely, three samples positive for IgA antibody to BP180-NC16a in serum and whole saliva were negative in parotid.

A description of the whole saliva results incorporating parotid saliva results are summarized (Table 3-6) and detailed here. Of the 30 whole saliva samples which were positive for IgA antibodies to BP180-NC16a (30/78), 12 had parotid saliva samples (12/30) while the rest did not (18/30) as parotid samples were randomly collected at the onset of the study. Of these 12, seven were positive in parotid and whole saliva (marked * in Appendix 3). Three were positive in serum and whole saliva (marked ^ in Appendix 3). One was positive in whole saliva alone (underlined in Appendix 3) and finally, one was positive in serum, whole and parotid saliva (marked + in Appendix 3). The remaining 18 samples which did not have parotid saliva samples analysed showed that 11 were positive in whole saliva alone (underlined in Appendix 3) while seven were positive in serum and whole saliva (marked ∨ in Appendix 3). No link was found between parotid saliva and clinical subgroups (pure oral, pure ocular or multisite disease) or with disease severity. Whole saliva was also tested using the anti-secretory component antibody and the results showed that 6/20 (30%) samples were positive, however, two were positive for SIgA in parotid saliva while the remaining four were negative for SIgA in parotid saliva.

a)



b)

Correlation between SIgA and IgA antibodies against BP180-NC16a in parotid saliva

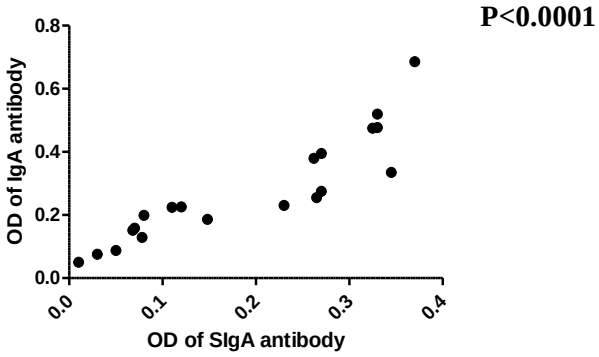


Figure 3-16 IgA and SIgA antibodies to BP180-NC16a in parotid saliva a) The figure represents the OD of SIgA antibody against BP180-NC16a in parotid saliva using an anti-secretory component antibody. The Y-axis represents OD while the X-axis shows the sample groups tested. Horizontal I bar indicates cut-off level (mean +2SD of the HC). Nine of twenty MMP patients (45%) were positive for SIgA tested by an anti-secretory component antibody which was also positive for IgA. b) Positive correlation between SIgA and IgA antibody in parotid saliva against BP180-NC16a (Spearman correlation r =0.954 p <0.0001). SIgA (Secretory IgA)

Table 3-6 Summary of IgA antibodies to BP180-NC16a in parotid and whole saliva

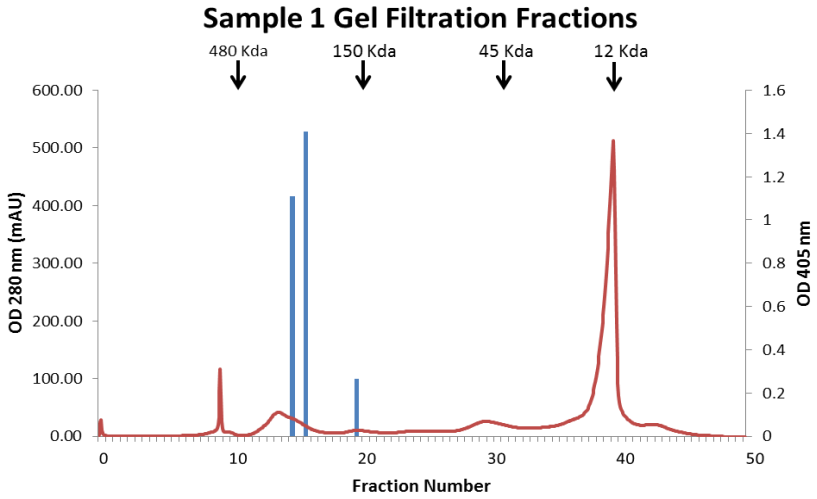
Summary of IgA antibodies to BP180-NC16a positivity in parotid and whole saliva	
Parotid saliva **9/20 +ve**	7 +ve P&WS (marked * Appendix 3) 1+ve P&S (marked × Appendix 3) 1+ve P&ws&S (marked + Appendix 3)
11/20 -ve	6 –ve in all P&WS&S 3 –ve P but +ve WS&S (marked ^ Appendix 3) 1 –ve P but +ve WS (underlined Appendix 3) 1 –ve P but +ve S (marked ° Appendix 3)
Whole saliva 30/78 +ve (38%) **12/30 +ve (had parotid samples analysed)**	7 +ve P&WS (marked * Appendix 3) 3 +ve WS&S (marked ^ Appendix 3) 1+ve P&ws&S (marked + Appendix 3) 1 +ve WS (underlined Appendix 3)
18/30 +ve (No parotid saliva analysed)	11 +ve WS (*underlined* Appendix 3) 7 +ve WS&S (marked ∨ Appendix 3)

+ve (Positive), -ve (Negative), P (Parotid saliva), WS (Whole saliva) and S (Serum)

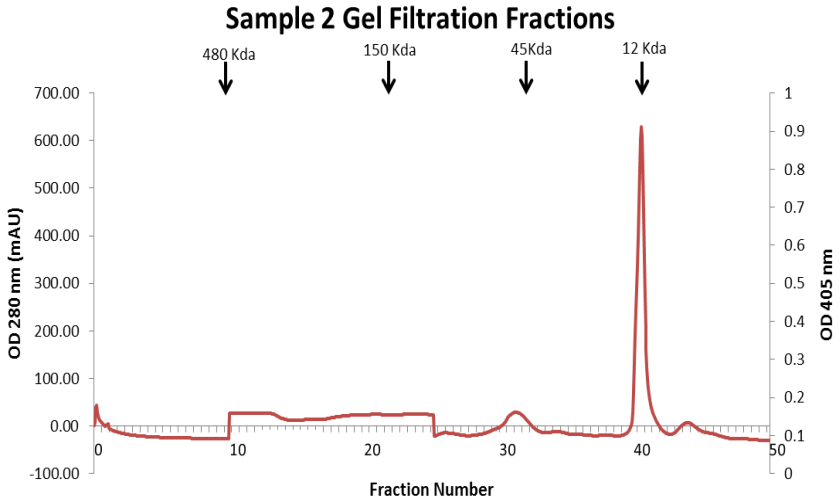
Whole saliva samples (from 4 patients) which were positive for SIgA antibody against BP180-NC16a by ELISA (Fig 3-17 a, c-e) and 1 whole saliva sample which was negative were fractionated by size exclusion chromatography (Fig 3-17 b). Secretory component and IgA were identified by Western blotting in fractions 14-17 (for all patients) corresponding to molecular weight ≥ 250 Kda and in one patient, additionally, fraction 20 corresponding to a molecular weight of approximately 170 Kda (Fig 3-17 a). Since secretory component has a molecular weight of approximately 80 Kda, these data indicate that secretory component is associated with a high molecular weight complex, presumably dimeric SIgA. The fraction with the molecular weight of 170 Kda could be as a result of some degradation which may correspond to monomeric IgA. Fractions in which secretory component and SIgA were detected by blots and control fractions (none

detected) were further tested on the BP180-NC16a pre-coated ELISA plates to investigate antibody activity against BP180-NC16a. As shown in (Fig 3-18), SIgA antibody to BP180-NC16a binding activity was associated with the presence of the high molecular weight form of secretory component.

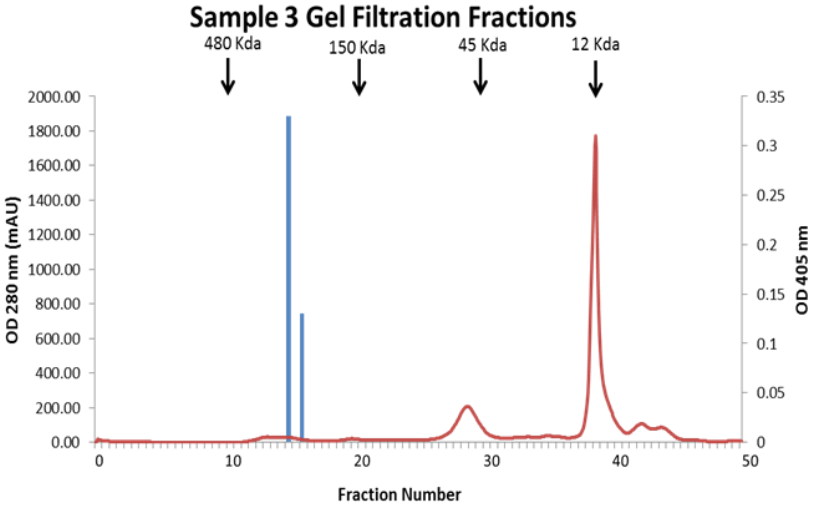
a)



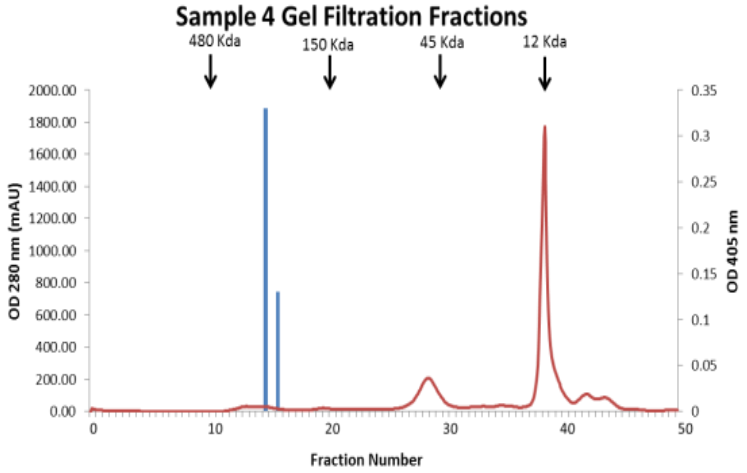
b)



c)



d)



e)

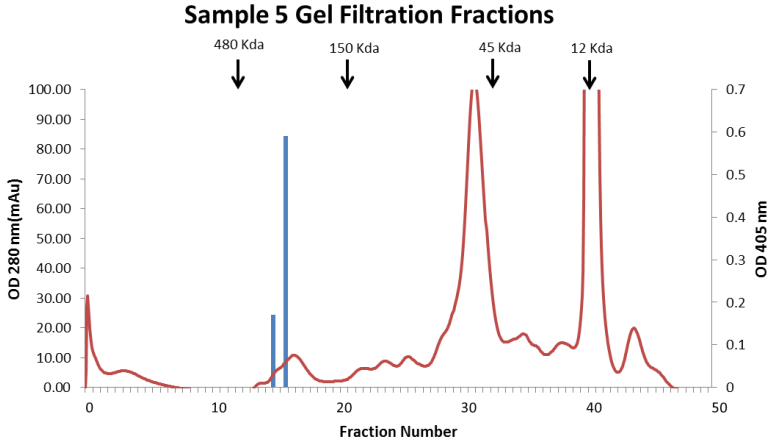


Figure 3-17 Anti-BP180 NC16a activity is associated with SIgA in parotid saliva
Following size exclusion chromatography of whole saliva, fractions were tested for binding to BP180-NC16a by ELISA (a,c-e) (b is a negative sample on ELISA) Chromatogram of whole saliva samples from patients with MMP. Absorbance shown in red (left axis) and reactivity of SIgA against BP180-NC16a in individual fractions shown in blue (right axis). The positions of the molecular weight standards are indicated

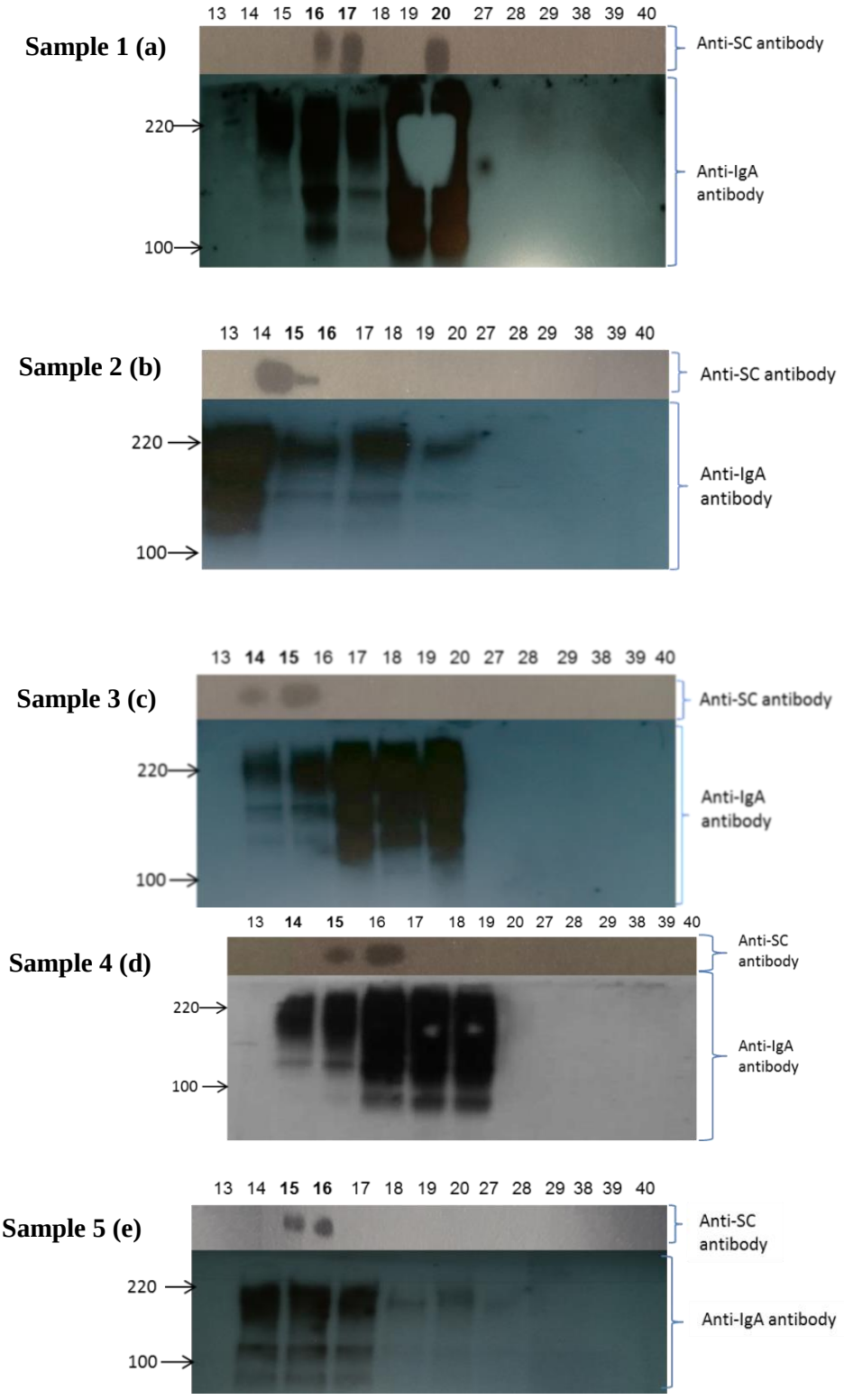


Figure 3-18 Western blotting of fractions from size exclusion chromatography showing results of probing with anti-secretory component antibody and probing with anti-IgA antibody

Parotid saliva samples were also tested for IgG and IgM antibodies to BP180-NC16a (n=20) and all were negative, confirming that the antibodies tested were of the IgA isotype (i.e. detection of SIgA) (Fig 3-19).

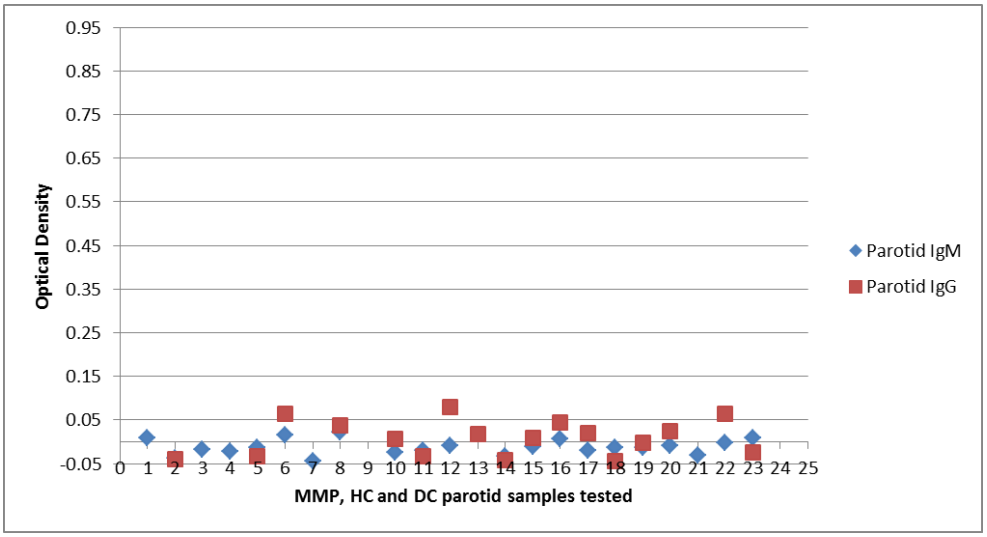


Figure 3-19 Parotid saliva samples in MMP, HC and DC tested for IgG and IgM antibodies to BP180-NC16a using pre-coated ELISA plates
The Y-axis represents optical density while the X-axis shows samples tested. Results show that both IgG and IgM antibodies were negative against the BP180-NC16a epitope using the anti-secretory component antibody further confirming the detection of SIgA. MMP samples (1-19), HC (20-21) and DC (22-23). HC (healthy control), DC (disease control), SIgA (secretory IgA).

Finally, the specificity of the anti-secretory component antibody used was tested using ELISA. Among serum samples positive for IgA antibodies to NC16a, (n=6) no reactivity was found using the anti-secretory component antibody while there were positive reactions with the anti-IgA antibody. This result confirmed that the anti-secretory component antibody was specific to secretory component only and not IgA (Fig 3-20).

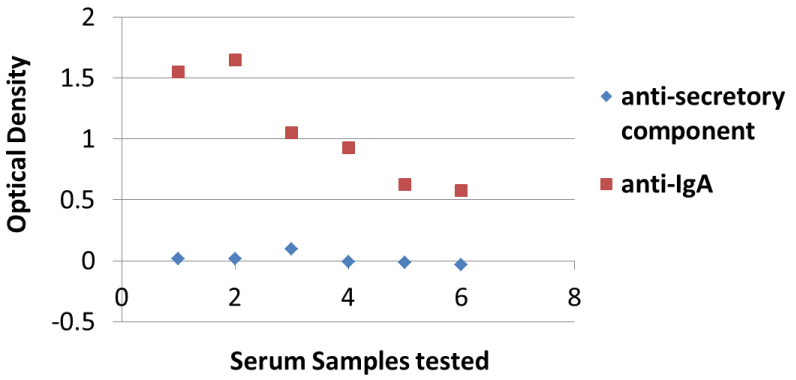
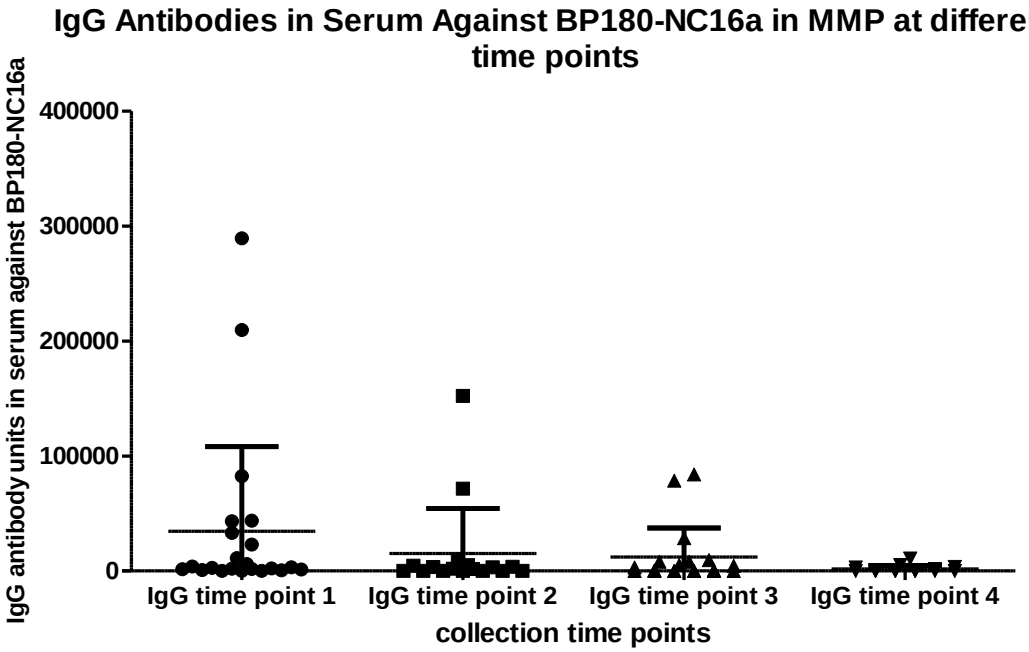


Figure 3-20 IgA antibody positive serum samples to NC16a showed no reactivity with anti-secretory component antibody A 96-well-plate was coated with anti-human IgA**,** serum sample starting 1:100 in 6 doubling dilution tested in duplicate and probed with anti-IgA antibody and anti-secretory antibody. Antibody activity using detection with anti IgA (red squares) and using anti secretory component (blue diamonds) in same samples

3.3 B) Sequential antibody titres in MMP patients to BP180-NC16a in relation to therapeutic response (Longitudinal Study 1b)

Samples of serum, whole and parotid saliva from MMP patients were collected sequentially. A total of four samples of each fluid were collected from 22 patients who were seen at 3 monthly intervals. The samples were tested against the commercially available BP180-NC16a pre-coated plates for IgG and IgA antibodies in both serum and saliva (whole and parotid). Oral disease severity scores were performed at each visit. Changes in antibody titre were seen in the majority of patients over nine months in serum (Fig 3-21 a-b) and whole saliva (Fig 3-22 a-b). Statistical analysis of the sequential samples showed that the change in serum IgG and IgA antibodies had a significant relationship with the change in severity scores (Spearman r=0.426 p=0.048) and (Spearman r=0.455 p=0.033) respectively (Fig 3-23 a-b). This indicates that the change in antibody level (titre), for both IgG and IgA in serum against BP180-NC16a, was significantly correlated with the change in oral severity score i.e. disease activity. The change in salivary antibodies (IgG and IgA), however, did not correlate significantly with the change in severity score. Notably, in the selected samples the titre dropped before the disease activity. Two representative graphs were plotted as an example of serum concentrations of antibodies to BP180-NC16a following therapeutic response and the oral disease severity score (Fig 3-24 a-b).

a)



b)

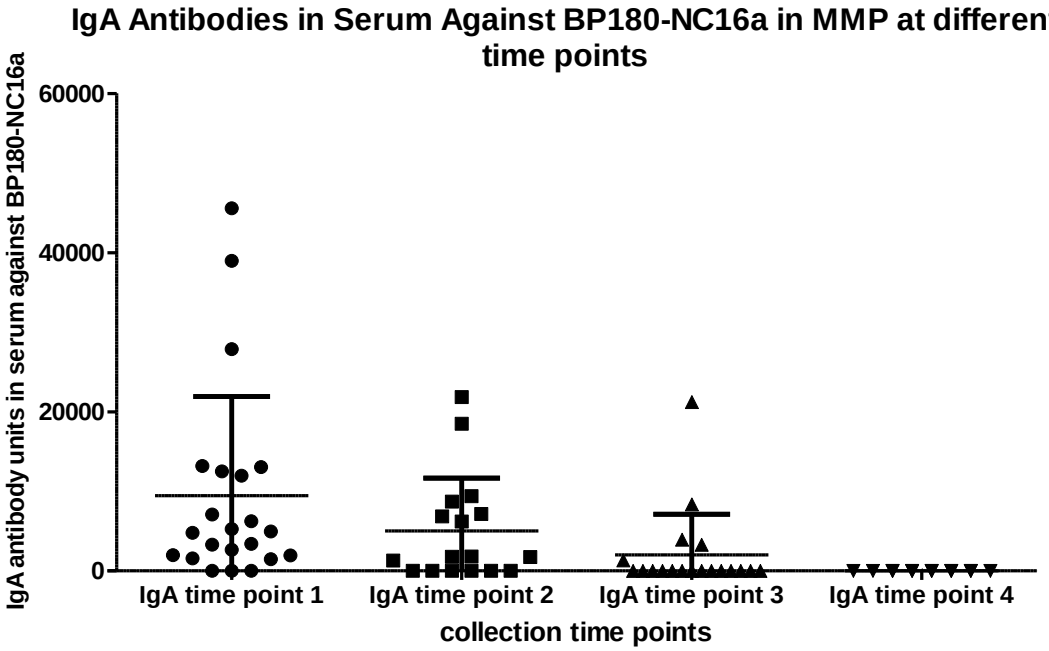
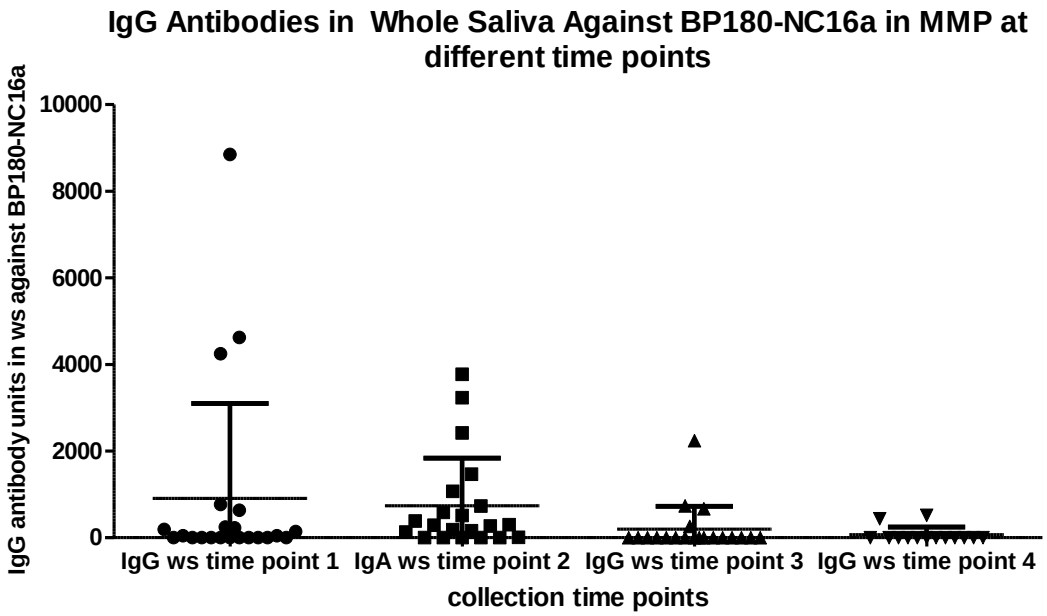


Figure 3-21 Levels of IgG and IgA antibodies to BP180-NC16a in serum in MMP patients at 3-monthly time-points. a) Anti-BP180-Nc16a IgG antibody level in serum at the different collected time points b) Anti-BP180-Nc16a IgA antibody level in serum at the different collected time points

a)



b)

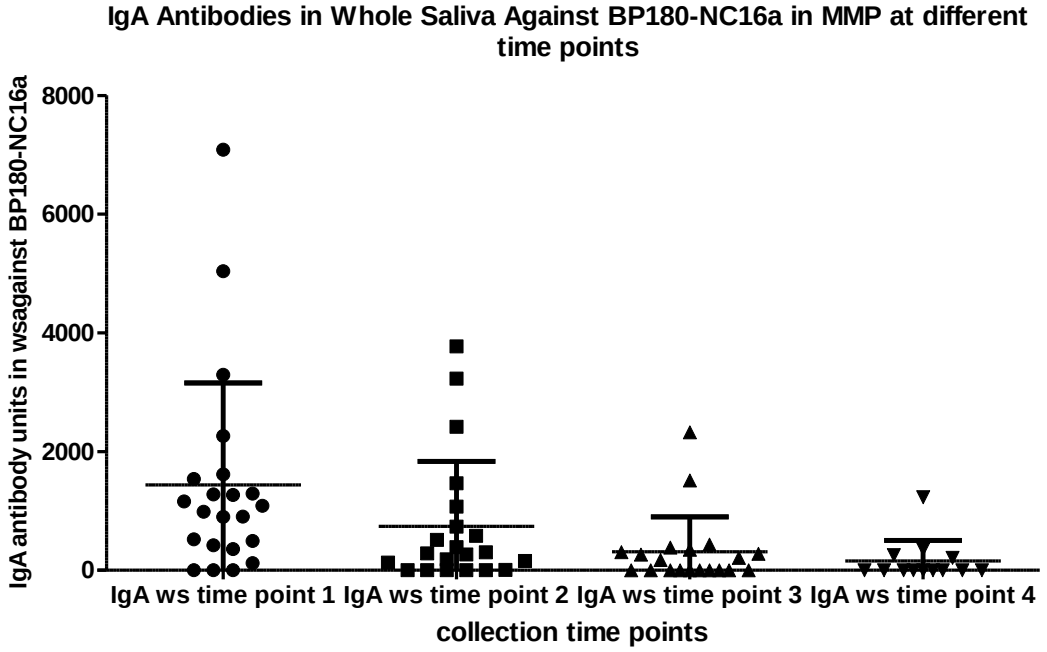
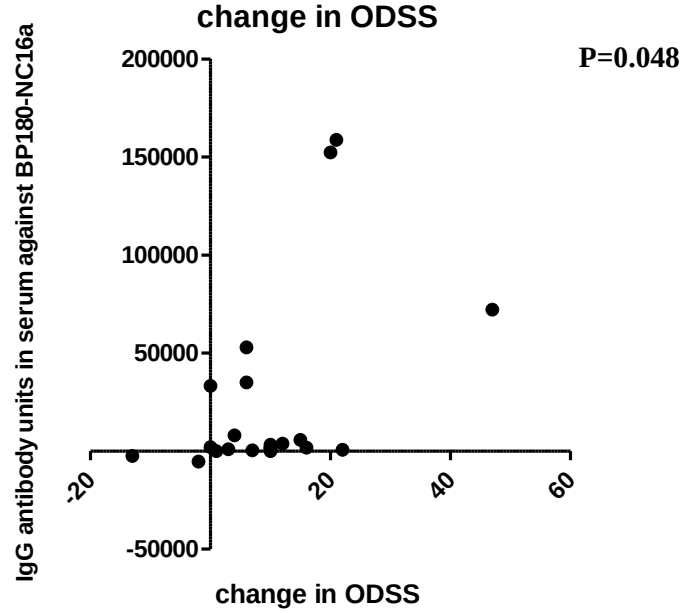


Figure 3-22 Levels of IgG and IgA antibodies to BP180-NC16a in whole saliva in MMP patients at 3-monthly time-points. a) Anti-BP180-Nc16a IgG antibody level in whole saliva at the different collected time points b) Anti-BP180-Nc16a IgA antibody level in whole saliva at the different collected time points

a) **Correlation between change in serum IgG antibodies to BP180-NC16a and change in ODSS**



b) **Correlation between change in serum IgA antibodies to BP180-NC16a and change in ODSS**

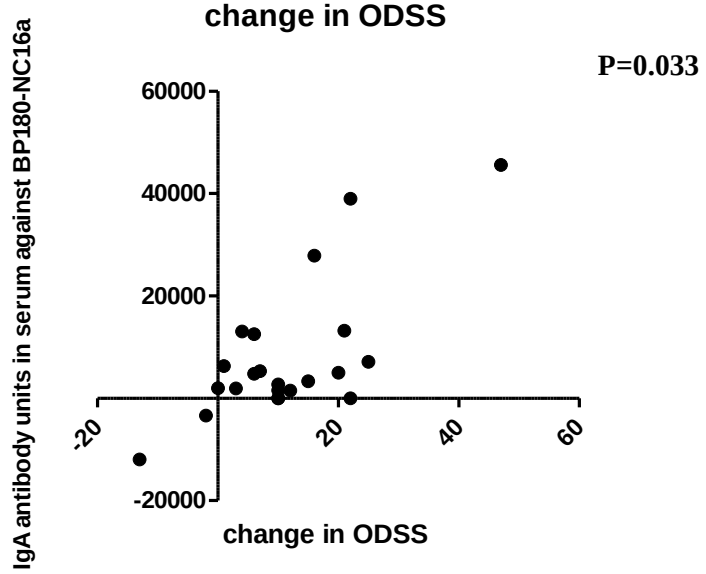
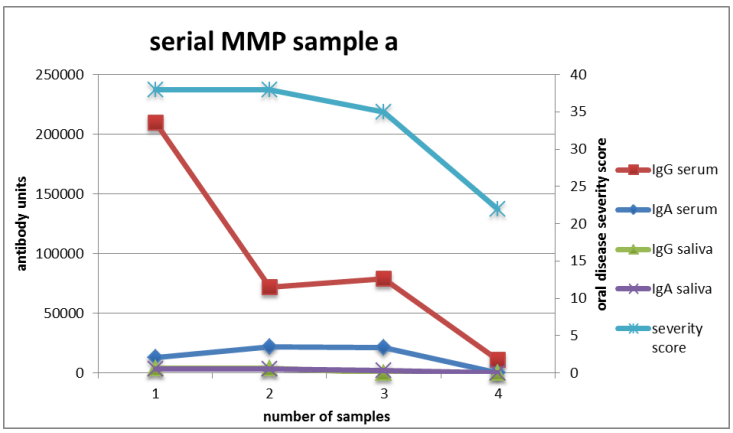


Figure 3-23 Correlation between the change in anti-BP180-NC16a antibody levels in serum (IgG/IgA) and the change in ODSS a) The change in anti-BP180-NC16a IgG antibody levels in serum and the change in oral disease severity score (ODSS) (Spearman r=0.426 p=0.048) b) The change in anti-BP180-NC16a IgA antibody levels in serum and the change in oral disease severity score (ODSS) (Spearman r=0.455 p=0.033)

a)



b)

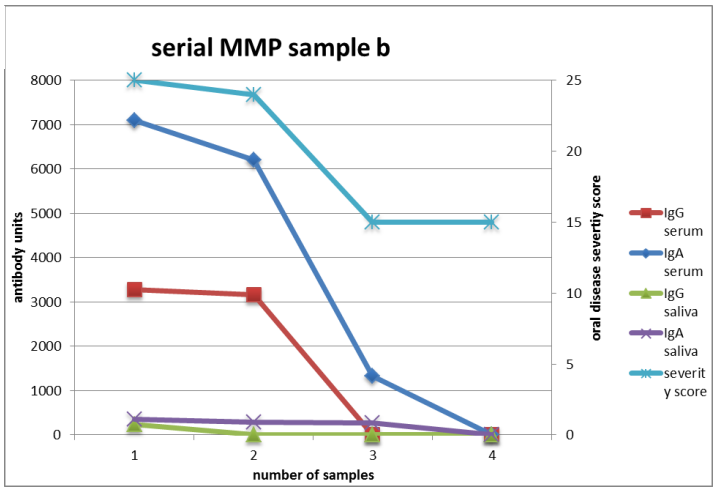


Figure 3-24 Graph showing serial MMP samples (2 representative samples) IgG and IgA units to BP180-NC16a in serum and whole saliva (ELISA) as well as oral disase severity score

The X-axis represents 4 serum or saliva samples which were collected at three monthly intervals. BP180-NC16a ELISA results for serum and whole saliva IgG and IgA antibodies, the **left** Y-axis (antibody units). The oral disease severity score, the **right** Y-axis (Oral disease severity score). Red (IgG antibody to BP180-NC16a in serum), Blue (anti-BP180-NC16a IgA antibody in serum), Green (IgG antibody to BP180-NC16a in whole saliva), Purple (anti-BP180-NC16a IgA antibody in whole saliva) and Turquoise (oral disease severity score)

3.4 Expression and purification of recombinant BP180-NC16a&BP180-4575

With the aim of developing ELISA procedures for analyses of autoantibodies directed against the 4575 epitope (residues1365 -1413) of BP180 (Zone et al., 1998) as well as NC16a, recombinant proteins fused to GST were expressed in *E. coli* and purified.

3.4.1 Sub-cloning and expression of GST-fusion proteins using pET15b

Plasmids pGEX-2TNC16a, pGEX-2T4575 and non-recombinant pGEX-2T were purified from cultures of transformed *E. coli*. DNA sequence analyses of the plasmids confirmed the presence of inserts encoding NC16a and 4575 epitopes (data not shown).

Recombinant fusion proteins as well as non-recombinant GST were expressed and purified by affinity chromatography using glutathione-Sepharose. Yields of proteins were consistently low at <500 µg/l culture. Use of an alternative expression vector was therefore investigated and DNA fragments (encoding GST, GST-NC16a and GST-4575) were sub-cloned into pET15b.

DNA fragments encoding GST-NC16a, GST-4575 and GST were amplified by PCR using the pGEX2T plasmids as templates. PCR-amplified products were analysed by agarose gel electrophoresis and bands corresponding to the expected sizes were evident (Fig 3-25).

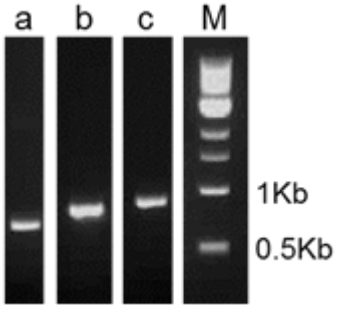


Figure 3-25 PCR-amplified DNA fragments of GST and GST-fusion proteins pGEX2T plasmids were used as templates for PCR-amplification of DNA fragments encoding GST and fusion proteins and aliquots of the reaction mixture were analysed by agarose gel electrophoresis and bands were visualised by staining with GelRed (0.1%). The composite figure shows amplification products: lane a. GST (expected size 778bp); b. GST-4575 (909bp); c. GST-NC16a (972bp). Marker (1Kb ladder) was loaded in lane M and the positions of the 0.5 and 1 kb bands are indicated

PCR amplified products were digested with *Nde*I and *Bam*HI ligated into pET15b (digested with the same restriction enzymes) and ligation products were used to transform *E. coli* XL-1 blue. DNA sequence analyses of purified plasmids from selected transformants confirmed sub-cloning of the respective inserts. Expression of GST and recombinant fusion proteins using pET15b as expression vector results in the insertion of a $(His)_6$ affinity tag at the N-terminus which allows purification by Ni^{2+}-affinity chromatography. Proteins were therefore purified by a 2-step procedure involving affinity chromatography with glutathione-Sepharose followed by affinity chromatography with Ni^{2+}-chelating Sepharose.

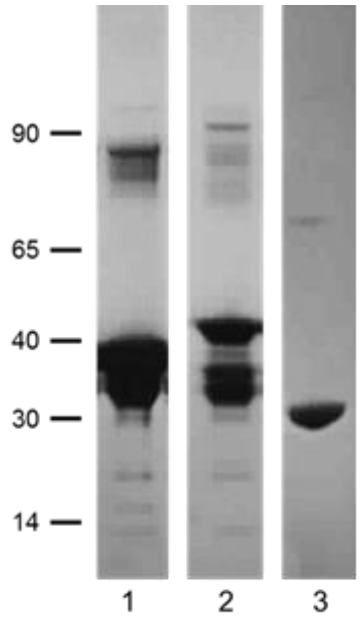


Figure 3-26 SDS-PAGE analysis of GST and GST-fusion proteins expressed using pET15b
Purified proteins were analysed by SDS-PAGE and visualised by staining with Coomassie blue. The composite figure shows: lane 1, GST-NC16a; lane 2, GST-4575; lane 3, GST. The positions of the molecular weight markers ($x10^{-3}$) are shown on the left

Purified proteins were analysed by SDS-PAGE as shown in (Fig 3-26). Bands of the expected molecular weights (36.8 kDa, GST-NC16a; 33.6 kDa, GST-4575; 29.2kDa, GST) were observed together with some bands of both higher and lower molecular weights. Western blotting with an anti-GST antibody confirmed that all bands were recombinant proteins (Fig 3-27). Some degradation resulting in lower molecular weight forms is evident while bands of higher molecular weight may represent dimeric forms. The identity of the recombinant proteins was further confirmed by mass spectrometric analyses of tryptic digests of excised bands which identified GST peptides as well as peptides from NC16a and 4575 epitopes. Use of the pET expression system resulted in levels of protein expression that were much higher than previously (in the range of 10 – 20 mg/l of culture).

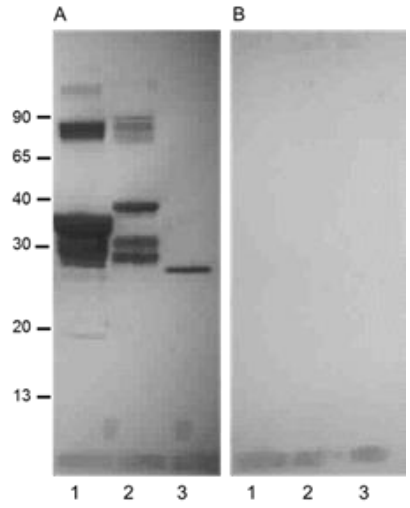


Figure 3-27 Western blotting of purified recombinant proteins Purified proteins (loaded as in the previous figure) were separated by SDS-PAGE and transferred to nitrocellulose filters for probing with anti-GST antibody (panel A). In the control filter (panel B), anti-GST antibody was omitted

3.4.2 Use of recombinant proteins in ELISA

Optimization of coating with the GST fusion proteins for use in ELISA was investigated by testing different concentrations and measuring binding of a positive (from previous ELISA determinations) MMP serum sample as shown in (Fig 3-28). A concentration of 1.5 µg/ml appeared to be optimal coating concentration. This was repeated for all 3 proteins (GST, NC16a and 4575). Results shown are for GST-NC16a.

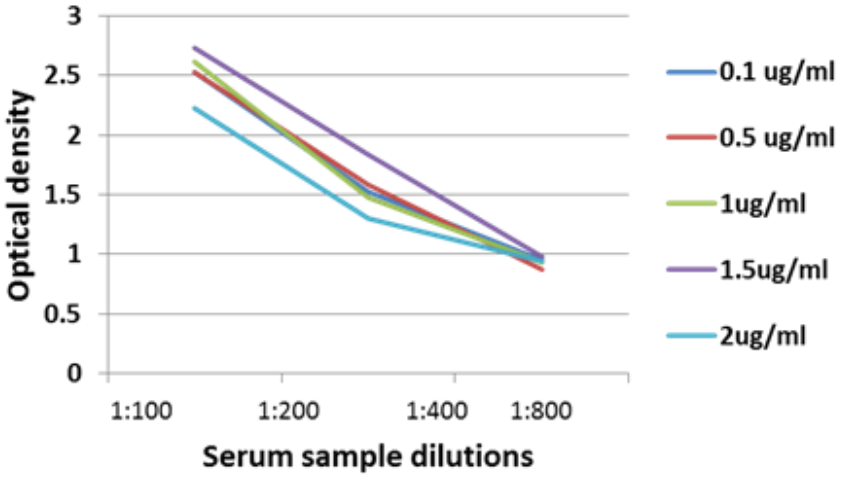


Figure 3-28 GST-BP180-NC16a ELISA plate coating optimization Different protein concentrations were tested against a positive serum sample and results were reported by optical density read at 405 nm

In further testing, serum binding to wells coated with GST-NC16a was compared with binding to GST. As shown in (Fig 3-29), a high level of serum binding to GST (with no fusion partner) was observed. Binding of antibody to the GST moiety of the NC16a fusion protein may therefore contribute to the OD values shown in (Fig 3-28). Pre-absorption of the patient's serum with lysate of *E. coli* BL21 expressing GST was investigated as a means of reducing the "background" binding to GST. The absorbed serum shows some decrease in binding (approximately 27%) to GST compared with non-absorbed serum (Fig 3-30 a-b).

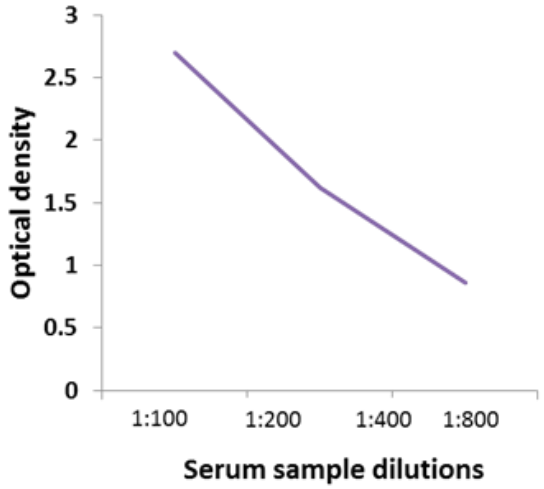
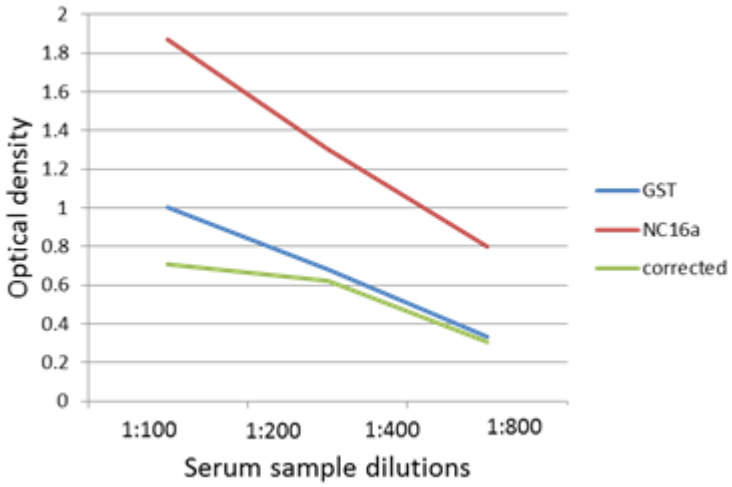


Figure 3-29 Serum binding to GST A high level of serum binding to GST (with no fusion partner) was observed

Although the recombinant proteins were purified by a double affinity chromatography procedure, it is possible that some *E.* coli-derived material may be present in the preparations and that this could contribute to apparent serum reactivity. The preparations were therefore further purified by size exclusion chromatography and tested again as coating antigens in ELISA. A decrease of 50% in serum binding to GST was noted with these preparations. Subsequently, a positive MMP sample and a negative MMP sample (as determined by ELISA using the commercially available pre-coated NC16a ELISA plates) together with healthy and disease controls were used to test an ELISA plate coated with the further purified peptides. There was no difference in the reactivity against NC16a-GST or 4575-GST between any of the MMP samples, whether they were positive or negative, and between the controls, both healthy and disease controls (Fig 3-31 a-c).

a)



b)

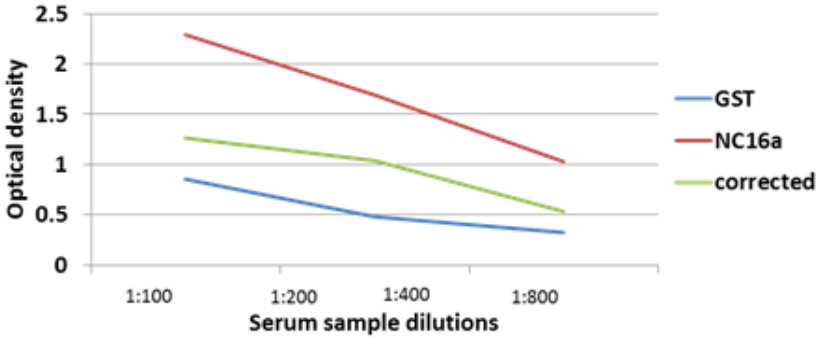
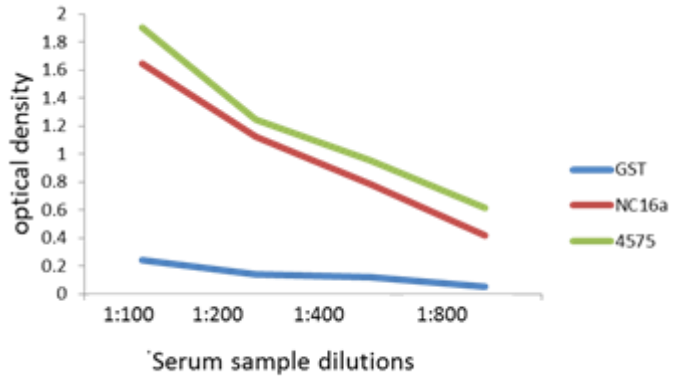


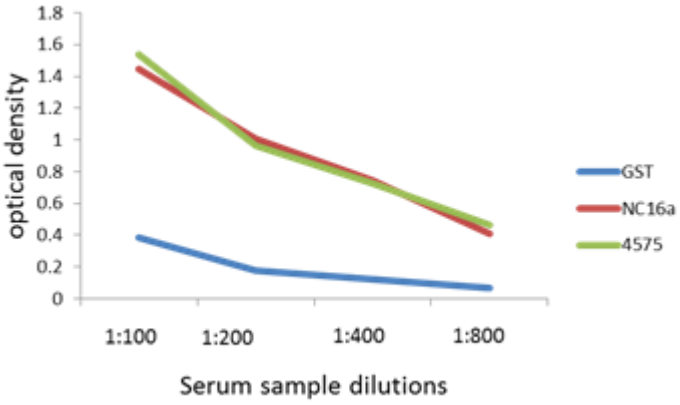
Figure 3-30 Reduced optical density of serum reactivity to GST after pre-absorbing the MMP serum sample with GST lysate

a) A positive MMP serum sample was tested against a coating of GST and NC16a without lysate pre-absorption b) A positive sample was tested after pre-absorbing the sera with the GST lysate and a reduction in optical density can be seen. GST (blue), NC16a (red) and corrected NC16a (NC16a – GST OD) (green)

a)



b)



c)

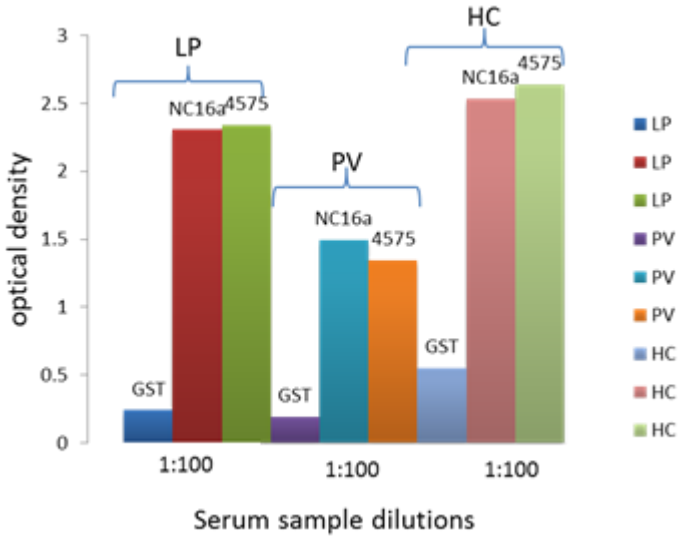


Figure 3-31 Testing MMP, healthy and disease control serum samples by ELISA against purified recombinant proteins ELISA plate coated with GST, GST-NC16a and GST-4575 after further purification by size exclusion chromatography. a) Positive MMP sample b) negative MMP sample both resulting in similar OD (tested in 4 doubling dilutions 1:100, 1:200, 1:400 and 1:800). c) Healthy control (HC) and disease controls; LP (Lichen planus) PV (Pemphigus vulgaris) resulting in OD as high as MMP samples (tested in duplicates 1:100). There was no discrimination by ELISA testing between the different samples; positive MMP, negative MMP, healthy control and disease control (LP and PV). All samples were pre-adsorbed with GST lysate. OD (optical density)

3.5 Discussion

In this study, whether IgG and IgA antibodies to the NC16a domain of BP180 could be detected by ELISA in both the serum and saliva of MMP patients and whether these biomarkers could be used in the analysis of disease severity and therapeutic response was explored. Moreover, whether there was an association with carefully defined clinical subgroups was investigated. This novel data has shown that whole saliva samples were reactive to the NC16a domain of BP180 with IgG antibodies in 14% of patients , IgA antibodies in 38% and either overall in 44% of the patients. In addition, locally produced secretory IgA antibodies (SIgA) to BP180-NC16a have also been demonstrated for the first time in saliva of these patients.

MMP antibodies in serum

In this study ELISA assays have been used to detect levels of IgG and IgA antibodies in both saliva and serum to the NC16a domain of BP180. The data has shown that MMP sera were reactive to the NC16a domain of BP180, with IgG antibodies in 36%, IgA antibodies in 28% or overall in 48% of the patients. These results are comparable to previously published work where 8/18 (44%) of patients were positive to IgG antibodies against the NC16a domain of BP180 on ELISA (Calabresi et al., 2007, Murakami H. et al., 1998). Similarly, data from collaborators has shown that the reactivity to the C-terminal domain (BP180-4575) on Western blot in which 7/39 (18%) MMP patients were positive for IgG antibody to BP180-4575, and 8/39 (21%) MMP patients were positive for IgA antibody to BP180-4575 was similar to those reported by Murakami where 30 % were positive (Murakami H. et al., 1998).

The results however, showed a lower percentage of reactivity with IgG antibody to the C-terminal domain of BP180 (4575) than previous studies positive in about 50% (Schmidt and Zillikens, 2013, Hayakawa et al., 2014).

Furthermore, 6/39 (15%) showed IgG antibody reactivity against laminin 332 (work undertaken by collaborators) which corresponds to previous literature and our collaborators own published results (Schmidt and Zillikens, 2013, Hayakawa et al., 2014).

When the cohort was subdivided into clinical phenotypes i.e. pure oral, pure ocular and multisite disease, results showed that IgG antibody to BP180-NC16a was highest in

both the serum (46% 23/50) and whole saliva (18% 8/50) of multisite disease patients. This was supported by finding a positive correlation between the number of sites in the multisite disease group and the IgG antibody titre in serum (p =0.0174) and whole saliva (p =0.0198) to BP180-NC16a. This suggests either that antibodies reflect the greater degree of antigen challenge found in multisite patients or that antibodies of a certain titre and avidity may result in multisite disease.

Interestingly, IgG antibody reactivity to BP180-NC16a was not detected in the pure ocular group. Contrarily, this group of patients had the highest ***IgA*** reactivity to BP180-NC16a in both serum 6/16 (38%) and whole saliva 7/16 (44%) when compared with the pure oral and multisite disease subgroups. In the literature, a link between the pure ocular subgroup and β4 integrin reactivity has been proposed (Chan, 2012). These results suggest a connection between pure ocular MMP and IgA reactivity against BP180-NC16a. A recent finding of IgA antibody to the C-terminal domain of BP180 has been reported (Solano-López et al., 2014). In this cohort one pure ocular patient was associated with IgA antibodies to the 4575 domain of BP180. Pure ocular pemphigoid patients generally have lower autoantibody levels detected, indeed DIF is reported as negative in 14-40% patients (Thorne et al., 2004, Bernauer et al., 1994, Kirzhner and Jakobiec, 2011).

The relatively low positivity of circulating autoantibodies in the series (46%), in addition to low numbers showing circulating antibodies in the pure ocular group (38%), may in part be due to the majority of patients being on long-term treatment and thus having low levels of disease activity as has been shown elsewhere (Sami et al., 2002).

It is also well recognised that MMP patient sera target one or more additional basement membrane zone (BMZ) antigens (Oyama et al., 2006). Setterfield et al detected serum IgG and IgA antibodies in MMP patients by IIF on salt-split skin resulting in 83.6% and 61.2% positivity respectively (Setterfield J. et al., 1998). Since IIF corresponds to reactivity against any or many of the BMZ components, this may explain the higher positivity with their technique. Nonetheless, IIF results for this study show a 35% positivity which is similar to our ELISA results. Using a combination of ELISA plates including additional epitopes of BP180, laminin 332, α6 and β4 integrin may significantly increase the diagnostic sensitivity of serum.

In this study, sequential samples have shown that the change in antibody levels of both IgG and IgA targeting BP180-NC16a in serum correlated significantly with an improved clinical severity (i.e. the therapeutic response to treatment) (Spearman

r=0.426 p=0.0482) and (Spearman r=0.455 p=0.0334) respectively. This was also shown by Setterfield et al and confirmed in a recent study (Di Zenzo et al., 2014, Setterfield et al., 1999).

MMP Antibodies in saliva

It has been proposed that in comparison to blood, saliva may provide more sensitive and specific biomarkers for oral diseases (Wong, 2006). Salivary diagnostics have been reported throughout the literature and have advanced dramatically as they are being used in the diagnosis of various conditions, including cancers (Arif et al., 2015, Cheng et al., 2014).

Since MMP predominantly affects the oral cavity in many patients, saliva may be a more sensitive medium. IgG antibodies are present in saliva as a serum transudate from gingival crevicular fluid, mucosal inflammation or via an ulcer and therefore the detection of these is likely to relate to the oral disease activity. Furthermore, it is has been shown that the amount of IgG in saliva as a transudate from the gingival crevice, is related to the degree of inflammation in periodontal disease (Brandtzaeg, 2007). In pemphigus vulgaris a high anti-Dsg3 IgG antibody level in whole saliva has been demonstrated in 94% of untreated patients (Hallaji et al., 2010, Hosseein Mortazavi, 2015). IgA antibodies, in addition to transudate from serum, may additionally appear in saliva as secretory IgA (SIgA) (i.e. produced from within the glands) either from major or minor salivary glands.

To investigate whether SIgA antibodies were present, parotid samples were included in addition to whole saliva. IgG antibodies to BP180-NC16a were detected in (11/78) (14%) of the MMP cohort in whole saliva. As expected, IgG antibodies were not detected in parotid saliva. The low percentage positivity in whole saliva may reflect the fact that the majority of patients had well-controlled disease at the time of sampling; only 68% of the patients had a positive oral disease score. In addition, it is possible that factors such as the presence of mucins in saliva may reduce the sensitivity of the assay for antibody detection. It would be interesting to compare the specific IgG positivity at presentation in MMP patients with very active disease and then to correlate the titre with a disease severity score. As stated, patients with multisite disease had the highest amounts of IgG antibody to BP180-NC16a in both serum and whole saliva (46% and 18%, respectively). Therefore it was not surprising to find a positive relation between the amount of IgG antibody to BP180-NC16a in whole saliva and the oral disease severity score in this subgroup.

Detection of secretory IgA antibody

IgA antibodies were also detected in both whole ***and*** parotid saliva. In whole saliva IgA antibodies were found in 30/78 (38%) of MMP patients tested, which was higher than for serum 22/78 (28%). Parotid samples were tested in an attempt to differentiate between IgA as a serum transudate (detected as a monomer and IgA positive) from local production (mucosally produced i.e. secretion from the gland) (dimeric IgA and secretory component positive). Interestingly, antibodies in almost half of parotid samples were both IgA and secretory component positive. Size exclusion chromatography showed activity with a high molecular weight complex in four patients who had tested positive on ELISA. These results were also confirmed by testing for IgG and IgM antibody in parotid samples of these MMP patients and were not detected. In addition, IgA antibody positive serum showed reactivity to anti-IgA antibody alone but not with the anti-secretory component antibody.

The precise mechanism of how these antibodies have been stimulated has not been ascertained. However, BP180 is found in salivary gland basement membranes (Gonzalez et al., 2011). Although patients do not present with symptoms of salivary gland involvement, it is possible to speculate that subclinical damage to the gland may subsequently result in exposure of BP180 within the basement membrane of the gland, leading to the production of local IgA autoantibodies directed specifically against epitopes of BP180. Furthermore, germinal centres within salivary glands have been reported in Sjögrens disease patients, which may help explain where these mucosal antibodies are being produced (Stott et al., 1998, Szodoray et al., 2005, Le Pottier et al., 2009). Alternatively, there may be distant mucosal site stimulation; since MMP patients may have other mucosal sites affected e.g. conjunctiva, nasopharynx, genital or oesophagus (MacPherson et al., 2008).

In this series, 4/9 of the patients producing specific IgA in their parotid glands had either multisite involvement including the oral cavity, conjunctiva, genital and/or nasopharynx, whilst 3/9 showed only oral involvement at the time of sampling and the remaining 2/9 were pure ocular patients. A further possibility is that SIgA in whole saliva may be due to secretion from the other major salivary glands or perhaps more importantly, from minor salivary glands which are known to be the major source of SIgA in saliva as minor salivary glands would give rise to secretory component positive antibodies into whole saliva (Brandtzaeg, 2007). Minor salivary glands are distributed

throughout the oral mucosa. Injury to these sites may damage the minor salivary glands similarly exposing the target antigen and stimulating local antibody production thus inducing further lesions. This might possibly help to explain the propensity for oral lesions on the soft palate where there are many minor salivary glands. This maybe further supported by this data since 4/6 of whole saliva samples tested for SIgA were negative in parotid saliva.

While oral involvement in MMP is sometimes considered to be a mild form of the disease, lesions often take several months to heal. In addition, the detection of serum anti-BMZ antibodies is often low or absent in mild oral MMP (Carrozzo et al., 2004). Thus it is puzzling why the disease is sometimes so active. It is possible that locally produced autoantibodies found in one secretion may also be found in others (Mestecky, 1987). So in a common mucosal system, the antibodies detected in parotid saliva may, in part at least, be stimulated by disease activity at sites other than oral. Some of these patients have ocular involvement or genital involvement and so could be producing these secretory antibodies in other mucosal sites.

MMP antibody avidity

Indirect immunofluorescence results may be low in MMP (50-80%) (Schmidt and Zillikens, 2013). Some patients may have very active disease even though the levels of detected antibodies are low or absent. This might be explained by the **avidity** of antibodies to a certain target antigen. Expression and purification of GST fusion proteins with the NC16a and 4575 epitopes was undertaken with the aim of developing ELISA procedures for the 4575 epitope. Sub-cloning into the pET15b expression vector markedly improved the yield of recombinant proteins from approximately 0.5 mg/l bacterial culture to 10-20 mg/l culture. In initial experiments to establish ELISA procedures, recombinant proteins and GST (as control) were used as coating antigens to test sera previously identified as positive or negative for binding to NC16a (with the commercially available plates). In contrast to the previous findings, both patient and control sera showed similar binding activity to the fusion proteins as well as significant binding to GST (absorbance values of approximately 50% of those of the fusion proteins). The possibility that the presence of antibodies against GST in the sera was obscuring differences in binding to BP180 epitopes was investigated by pre-absorption of lysates of GST-expressing *E.coli*. While this reduced binding to GST in control wells, it did not improve discrimination between binding of patient and control sera to

the NC16a and 4574 fusion proteins. Thus it seems unlikely that recognition of the GST fusion proteins by both patient and control sera can be attributed to antibodies directed against GST.

GST-fusion proteins have been used extensively to investigate antibody binding to autoantigen epitopes and few report significant binding to GST alone. In analyses of serum immunoreactivity to an epitope of the human muscarinic acetylcholine receptor 3, expressed as a GST fusion protein, ELISA absorbances of sera from 40 healthy control patients were all below 0.2 while values for 33/40 patients were 0.3 or higher (Marczinovits et al., 2005). GST has been reported as an autoantigen in patients with glaucoma (Yang et al., 2001) where anti-GST antibodies were present sera of 52% of patients and 20% of age-matched control subjects. In a preliminary study, GST has been suggested as an autoantigen in multiple sclerosis with anti-GST activity reported in cerebrospinal fluid of 27% of patients compared with 25% of normal controls (Laffitte et al., 2005). Although this does not seem a strong case for identification of GST as an autoantigen, it does point to significant anti-GST activity in patients as well as in healthy controls. In further work, it may be appropriate to investigate alternative fusion partners for the NC16a and 4574 epitopes as well as investigating further means of reducing anti-GST serum immunoreactivity.

3.6 Conclusion

To my knowledge, this is the first study demonstrating both IgG and IgA antibodies to BP180-NC16a in saliva. There has only been one previous study testing salivary anti-BMZ antibodies in MMP patients. Andreadis et al, tested 12 MMP serum and whole saliva samples for IgG reactivity, using commercially available BP180-NC16a pre-coated ELISA plates (Andreadis D, 2006). They were unable to show IgG reactivity in either serum or whole saliva and the authors proposed that MMP patients produced autoantibodies that were reactive against the C-terminal domain of BP180 and not the NC16a domain. Although we and others have shown that both IgG and IgA autoantibodies may target the C-terminal domain of BP180, in this study the presence of autoantibodies in both serum and saliva against the NC16a epitope of BP180 has been demonstrated (Hayakawa et al., 2014, Lee et al., 2003, Murakami H. et al., 1998). Moreover, the results have also shown that ELISA testing of serum samples from MMP patients for IgG and IgA antibodies to BP18-NC16a can be related to oral disease activity and therapeutic response.

These novel findings have shown that not only are NC16a specific salivary IgA antibodies present in MMP patients, but that this medium may have a greater sensitivity for detecting IgA. Further investigation in a larger cohort including patients with untreated disease and using parallel mucosal secretions, e.g. tears, will be most informative. To further increase the sensitivity of saliva as a diagnostic and disease monitoring medium, the additional testing for reactivity with laminin 332, α6 and β4 integrin is likely to be most informative.

3.7 Summary

- Serum IgG antibodies against the NC16a domain of BP180 were detected in 28/78 (36%) of MMP patients
- Serum IgA antibodies were found in 22/78 (28%). Combined serum IgG and/ or IgA antibodies were positive in 38/78 (48%) patients
- IgG antibodies to BP180-NC16a were detected in 11/78 (14%) of MMP whole saliva samples shown for the first time
- IgA antibodies to BP180-NC16a were detected in 30/78 (38%) of MMP patients in whole saliva. Combined whole saliva specific IgG and/ or IgA was positive in 34/78 (44%). Shown for the first time and as sensitive as serum IgA detection
- Both SIgA and IgA antibody 9/20 (45%) were positive in parotid saliva samples which has not been reported previously
- There was a positive association between the presence of serum IgG antibodies to BP180-NC16a with multisite disease lesions ($X^2 = 11.9$ $p = 0.003$)
- A significant association was found between the presence of IgA antibody against BP180-NC16a in whole saliva and the presence of both oral and/or ocular disease ($X^2 = 6.23$ $p = 0.016$)
- Pure ocular MMP patients showed no IgG antibody reactivity against BP180-NC16a in either serum or saliva; however they were positive for IgA antibody to BP180-NC16a in serum and whole saliva (38% and 44%) respectively.
- The higher the number of sites involved in multisite MMP, the higher the IgG antibody titre in serum and whole saliva against BP180-NC16a ($p = 0.017$ and $p = 0.019$, respectively)
- Sequential samples showed that the change in serum antibodies (IgG and IgA) have a significant relation with the change in severity scores ($p=0.048$ and 0.033) respectively, following therapeutic response

4 Results MMP Study 2

Serum and salivary antibodies in MMP to alpha 6 beta 4 Integrin in relation to clinical phenotype and disease activity (Cross-sectional study)

4 Serum and salivary antibodies in MMP to alpha 6 beta 4 Integrin in relation to clinical phenotype and disease activity (Cross-sectional study)

4.1 Introduction

MMP is a heterogeneous disease with various target antigens within the basement membrane zone. BP180, which is considered the main target antigen, was studied in the previous chapter. In this section, α6β4 is the antigen of interest. It has been proposed that in oral and ocular pemphigoid, the target antigens are the α6 and β4 fragments respectively (Rashid et al., 2006a, Rashid et al., 2006b). However, other antigens including BP180, have been shown to be the most prominent target antigens in both subgroups (Hayakawa et al., 2014, Solano-López et al., 2014, Calabresi et al., 2007). Consequently, no specific antigen has been definitively associated with a site-specific phenotype as yet.

No study thus far has reported salivary antibodies against the α6β4 integrin. Since pure oral pemphigoid patients have been reportedly linked with serum antibodies to α6 integrin and since autoantibodies to BP180-NC16a domain could be detected in saliva as described in chapter 3, testing saliva samples from MMP patients for autoantibodies against α6β4 integrin was carried out.

The aims were to:

1) Identify the whether serum and saliva IgG and/or IgA autoantibodies to α6β4 were present in our cohort of MMP patients

2) Identify whether there was an association between autoantibodies against α6β4 integrin and carefully defined clinical subgroups

3) Determine antibody avidity using BIAcore technology

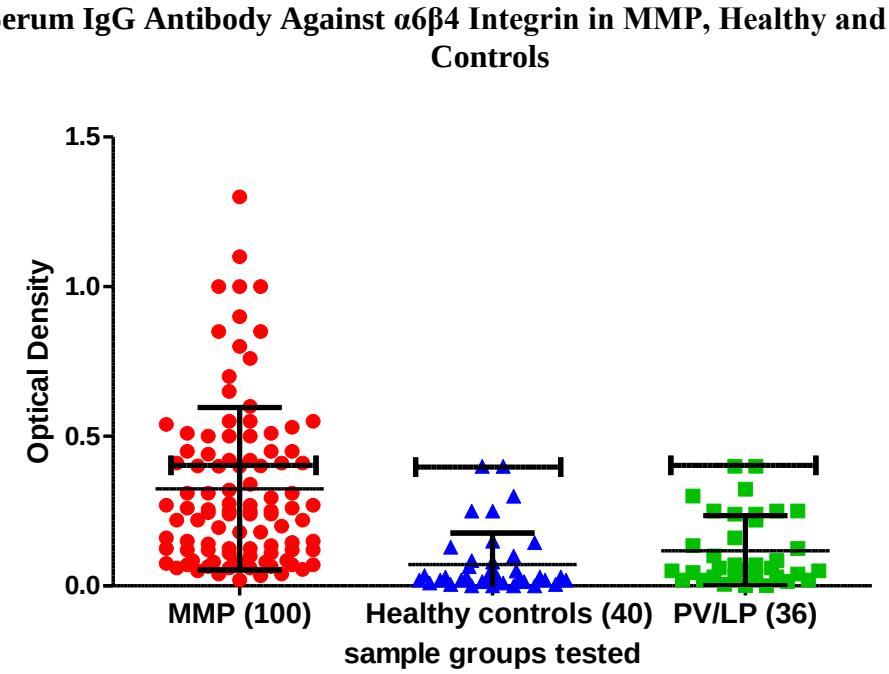
The cohort of MMP patients was subdivided into ‘pure ocular’, ‘pure oral’ and multisite disease and the results were further analysed. The presence of serum or saliva antibodies against α6β4 integrin was determined by ELISA using a soluble form (extracellular domains) of the integrin. Western blotting was investigated as a means of differentiating between α6 and/or β4 reactivity in antibody positive patients. Surface plasmon resonance (spr)-based assays were also investigated as a potential means to measure avidity of autoantibodies.

4.2 Serum, whole and parotid saliva

In this study, matched serum and whole saliva was tested using ELISA plates which were coated using a human recombinant α6β4 integrin (see Chapter 2: Materials and methods). Samples from one hundred MMP patients, forty healthy controls (HC) and 36 Disease controls (DC) (including 15 LP and 21 PV) were tested. The mean IgG antibody values in serum (0.325±0.27) and in whole saliva (0.16±0.18) were significantly different than those of healthy controls in serum (0.07±0.11) and in whole saliva (0.08± 0.07) by using one-way ANOVA ($p<0.0001$). Values greater than the mean +2 SD of the healthy controls were considered positive.

The results using ELISA showed that 36/100 (36%) of MMP patients were positive for IgG antibody to α6β4 in serum and 18/100 (18%) of MMP patients were positive for IgG antibody to α6β4 integrin in whole saliva (Fig 4-1 a-b) (Table 4-1). Oral disease was clinically present in 74/100 (74%) of patients, all of whom had active disease at the time of blood and saliva sampling with oral disease severity scores ranging from 5-43 (see Appendix 3). IgA antibody to α6β4 in serum, whole or parotid saliva was not detected (Fig 4-2).

a)



b)

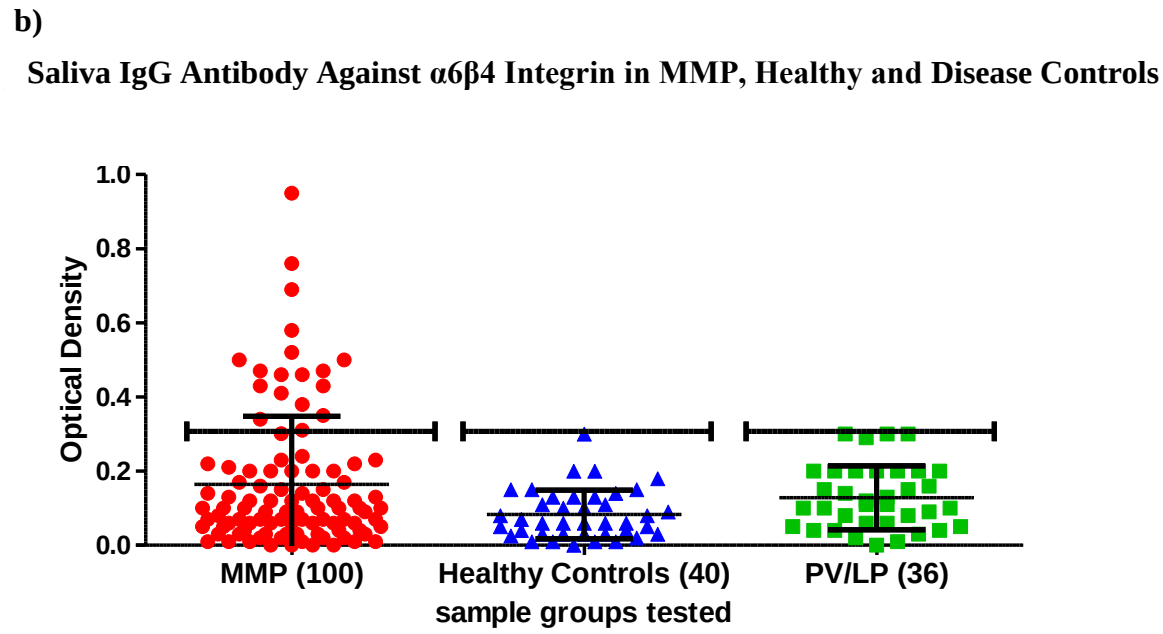
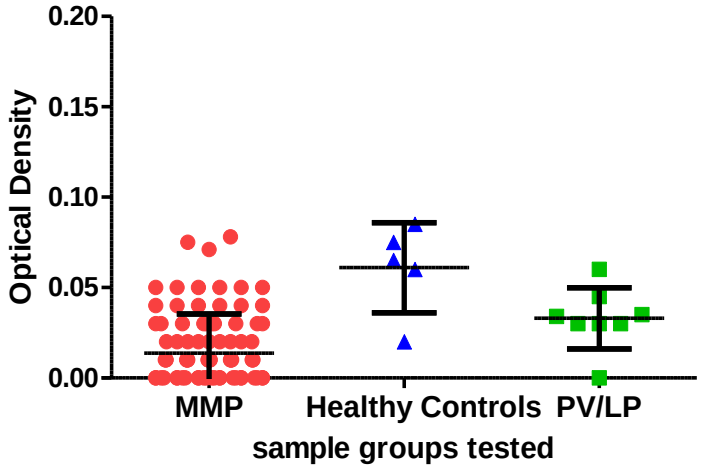


Figure 4-1 IgG antibody against α6β4 integrin in serum and whole saliva in MMP patients, healthy and disease controls using ELISA (mean +/- SD) The Y-axis represents optical density values while the X-axis shows the sample groups tested. Values greater than the mean + 2SD of the HC were considered positive. The horizontal I bar represents the cut-off. a) Serum IgG antibody against α6β4 integrin showed 36/100 (36%) positivity. b) Whole saliva IgG antibody against α6β4 integrin showed 18/100 (18%) positivity

a)

Serum IgA Antibody Against α6β4 Integrin in MMP, Healthy and Disease Controls



b)

Saliva IgA Antibody Against α6β4 Integrin in MMP, Healthy and Disease Controls

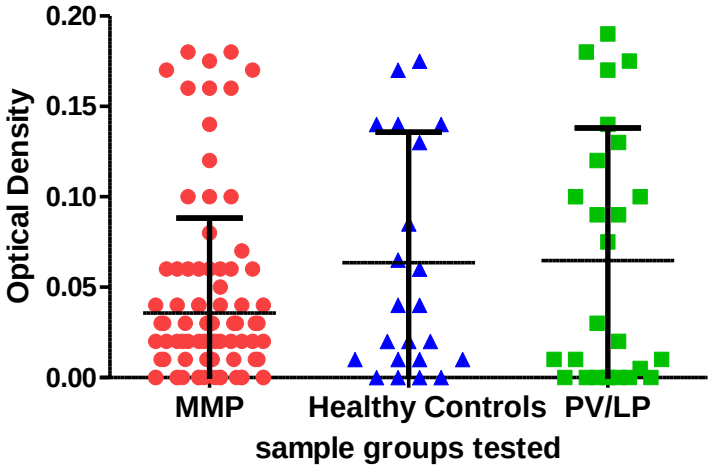


Figure 4-2 IgA antibody against α6β4 integrin in serum and whole saliva in MMP patients, healthy and disease controls using ELISA (mean +/- SD) The Y-axis represents optical density values while the X-axis shows the sample groups tested. a) Serum (MMP n=50, HC n=5, DC n=8) b) Whole saliva (MMP n=100, HC n=22, DC n=26). There was no significant difference between the Means of IgA antibody level in all three subgroups tested. Therefore, IgA antibody to α6β4 integrin was not considered diagnostic

Table 4-1 Study 2 ELISA results for IgG antibodies to α6β4 integrin in serum and whole saliva

Study 2 MMP (n=100)	IgG Serum	IgG Whole Saliva
ELISA Results against α6β4	36/100 (36%)	18/100 (18%)

A positive correlation was found between IgG antibody in serum and whole saliva to α6β4 integrin (Spearman r =0.493 p <0.0001) (Fig 4-3). There was also a positive relationship between IgG antibody in whole saliva against α6β4 integrin and oral disease severity score (Spearman r =0.276 p =0.0055) (Fig 4-4).

Correlation between serum and whole saliva IgG antibodies against α6β4 integrin

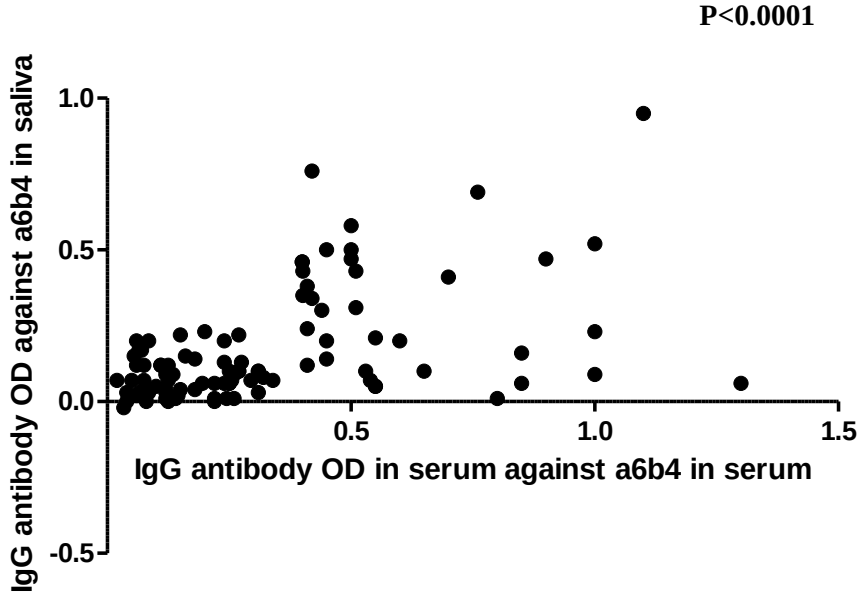


Figure 4-3 Correlation between IgG antibodies to α6β4 integrin in serum and whole saliva in MMP patients (Spearman r =0.493 p <0.0001) OD =optical density

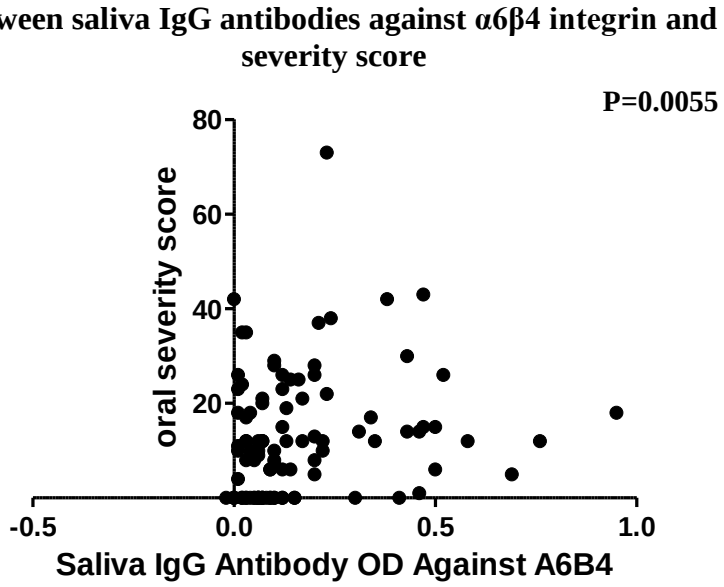


Figure 4-4 Correlation between IgG antibody in whole saliva against α6β4 integrin and the oral disease severity score of MMP patients (Spearman r =0.276 p = 0.0055) OD =optical density

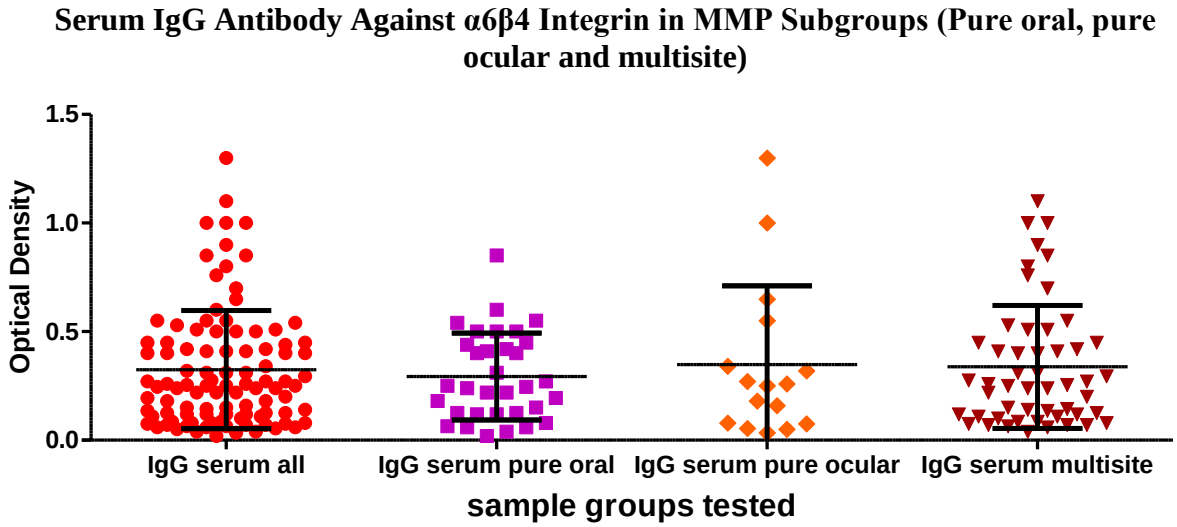
Relationship between antibodies and clinical phenotype

When clinical subgroups were analysed, the results for serum showed that in pure oral MMP 14/33 (42%) were positive for IgG antibody to α6β4 integrin. In the pure ocular subgroup, 4/16 (25%) were positive, while the multisite subgroup showed 18/51 (35%) positivity (Fig 4-5 a). A positive correlation between IgG antibody in serum against α6β4 and the oral disease severity score in the pure oral subgroup was found (Spearman r =0.365 p =0.037) (Fig 4-6).

For saliva, IgG antibody to α6β4 integrin was detected in 7/33 (21%) of pure oral patients, 11/51 (22%) of multisite disease and none of the pure ocular group (Fig 4-5 b) (Table 4-2). The multisite subtype had the highest percentage of positivity of IgG antibody to α6β4 integrin.

Clinical phenotype antibody distributions:

a)



b)

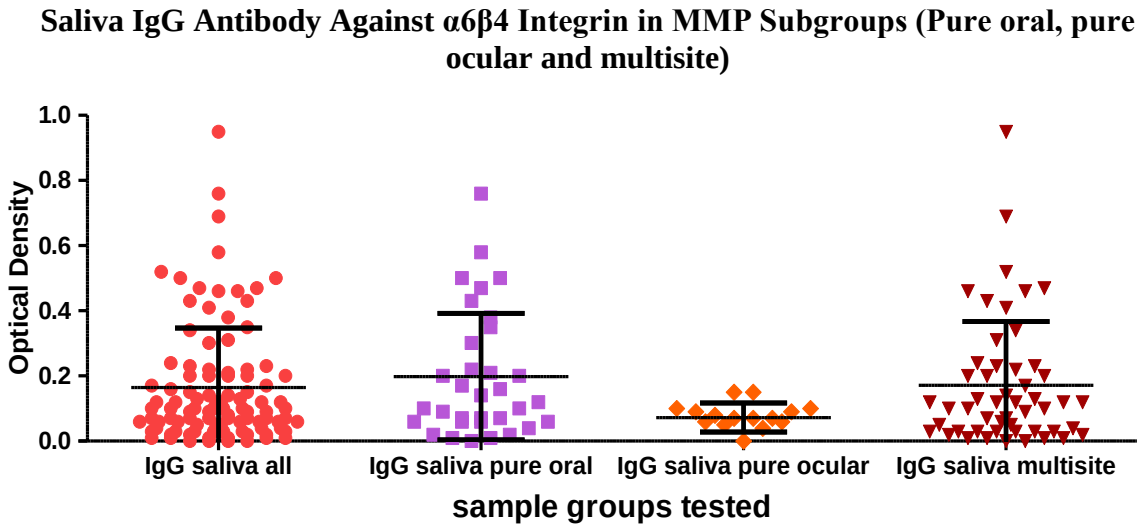


Figure 4-5 MMP patients subgrouped according to clinical phenotype and tested for IgG antibody against α6β4 integrin in serum and whole saliva (Mean +/- SD) Subgroups include MMP cohort (n=100), pure oral (n=33), pure ocular (n=16) and multisite disease (n=51). The Y-axis represents the OD values. The X-axis shows the sample groups tested. Positivity was established at values greater than the mean + 2SD of the HC group as previously stated. a) Serum IgG antibody against α6β4 was positive in 36/100 MMP (36%), pure oral 14/33 (42%), pure ocular 4/16 (25%) and 18/51 (35%) were positive in the multisite group. b) Whole saliva IgG antibody against α6β4 were positive in 18/100 MMP (18%), pure oral 7/33 (21%), pure ocular none were positive (0/16) and 11/51 (22%) were positive in the multisite group

Correlation between Serum IgG antibodies against α6β4 integrin and oral disease severity score in Pure Oral MMP

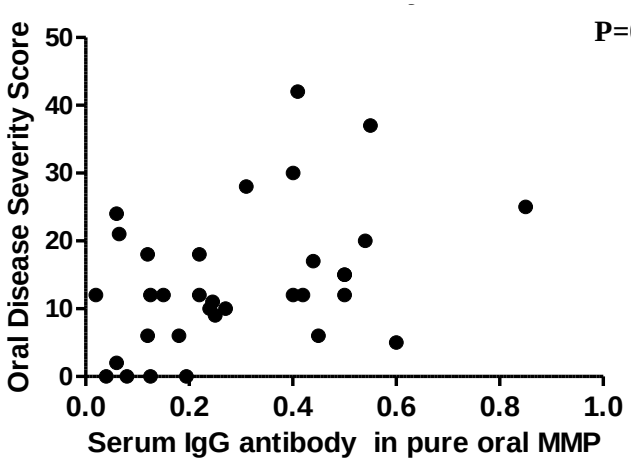


Figure 4-6 Correlation of IgG antibody OD against α6β4 in serum in pure oral MMP subgroup and the oral disease severity score (Spearman r =0.365 p =0.037) OD =optical density

Table 4-2 Summary of IgG antibodies in serum and whole saliva against α6β4 ELISA by clinical phenotype distribution

Study by phenotype MMP (n=100) against α6β4 integrin	Pure Oral (n=33)	Pure Ocular (n=16)	Multi-site (n=51)
IgG Serum	14/33 (42%)*	4/16 (25%)	18/51 (35%)
IgG Whole Saliva	7/33 (21%)	0	11/51 (22%)*

***** represents the highest percentage value of autoantibody detected

4.3 Investigation of surface plasmon resonance for measuring binding of serum autoantibodies to α6β4 integrin

The α6β4 integrin (approximately 2,000 resonance unit -RU-) was covalently coupled to the surface of flowcell 2 (Fc2) of a sensorchip while Fc1 (no protein coupled) served as a reference cell. Anti-α6 or anti-β4 polyclonal antibodies (see Chapter 2, used in western blotting below as well) were injected separately over the sensorchip surface to confirm immobilisation of the integrin and exposure of at least some epitopes. As shown in Fig 4-7, both antibodies bound to the immobilised ligand. The anti-β4 antibody bound much more strongly with K_D (equilibrium dissociation constant) of approximately $3x10^{-10}$ M compared with the anti-α6 antibody that bound with K_D of approximately $1.6x10^{-7}$ M (Fig 4-7). In contrast, no binding was evident when patients' sera was injected over the sensorchip surface (Fig 4-8). The decrease in the spr signal is the result of changes in the refractive index of the solution due to the presence of serum components.

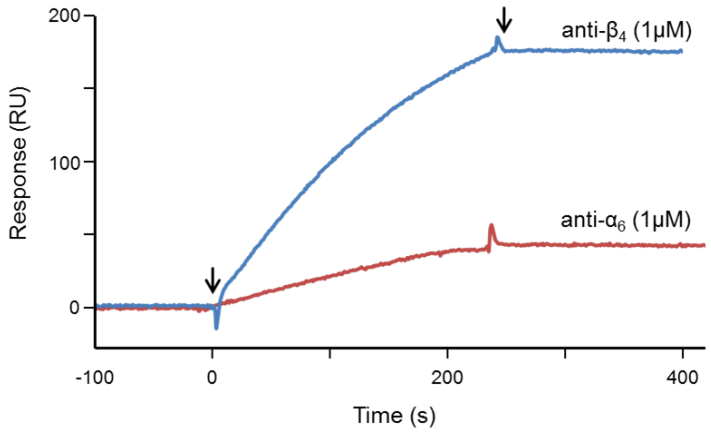


Figure 4-7 Binding of anti-α6 and anti-β4 antibodies to immobilised integrin Integrin (approximately 2,000 RU) was immobilised on the sensorchip surface of Fc2 while Fc1 served as a control surface. Superimposed difference sensorgrams show binding of each antibody. Arrows indicate the start and end of injection

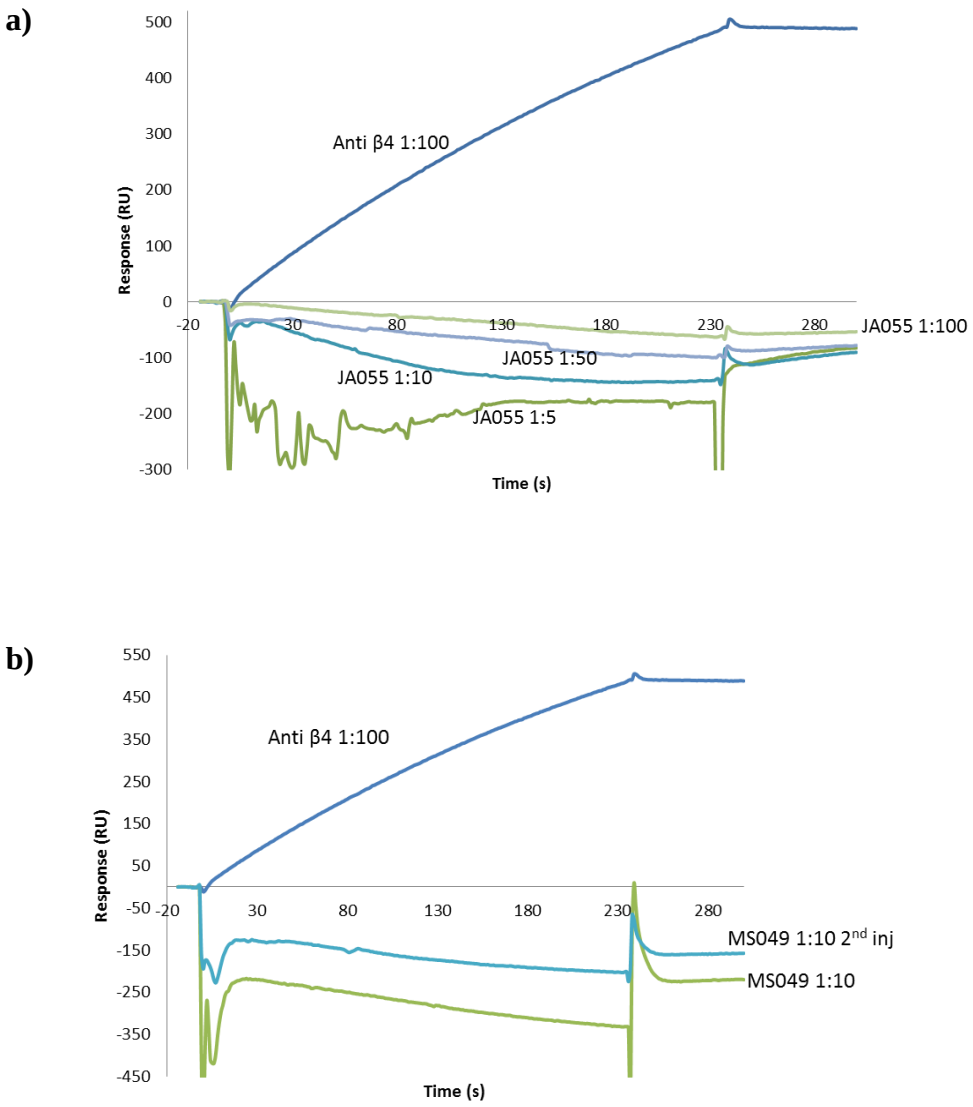


Figure 4-8 Superimposed sensograms depicting binding activity of autoantibodies to α6β4 integrin The Y-axis shows the response (in resonance units) and the X-axis is the time in seconds. α6β4 integrin was immobilized on the sensor chip of Fc2 while Fc1 was left blank. The resulting sensograms were calculated by subtracting the signal of Fc1 from Fc2. Sheep anti-β4 antibody (1μM) was injected over the sensorchip surface as control for antibody binding (dark blue). a) A serum sample (MMP JA055, positive on α6β4 ELISA) was loaded at four dilutions (1in 5, 1in 10, 1in 50 and 1in 100) b) A second serum sample (MMP MS049, positive on α6β4 ELISA) was loaded (1in10 dilution) and repeat sensorgrams are shown

4.4 Investigation of specificity of anti-integrin antibodies

The molecular weights of the α6 and β4 extracellular domains in the complex, used above for ELISA, are 125-135 kDa and 100-110 kDa, respectively (R&D Systems: https://www.rndsystems.com/products/recombinant-human-integrin-alpha-6-x1-beta-4-protein-cf_5497-a6). The two polypeptides were therefore separated by SDS_PAGE

and transferred to nitrocellulose filters for Western blotting. As shown in (Fig 4-9 a), identity of the separated polypeptides was confirmed by probing with anti-α6 or anti-β4 polyclonal antibodies (see Chapter 2) (lanes 1and 2 respectively) and (Fig 4-9 b lane 2).

Sera from nearly all patients as well as healthy controls appeared to react with the α6 polypeptide while little or no reactivity towards the β4 polypeptide was evident (Fig 4-9). In controls where serum (or first antibody) was omitted, no bands were observed (Fig 4-9 b lane 1).

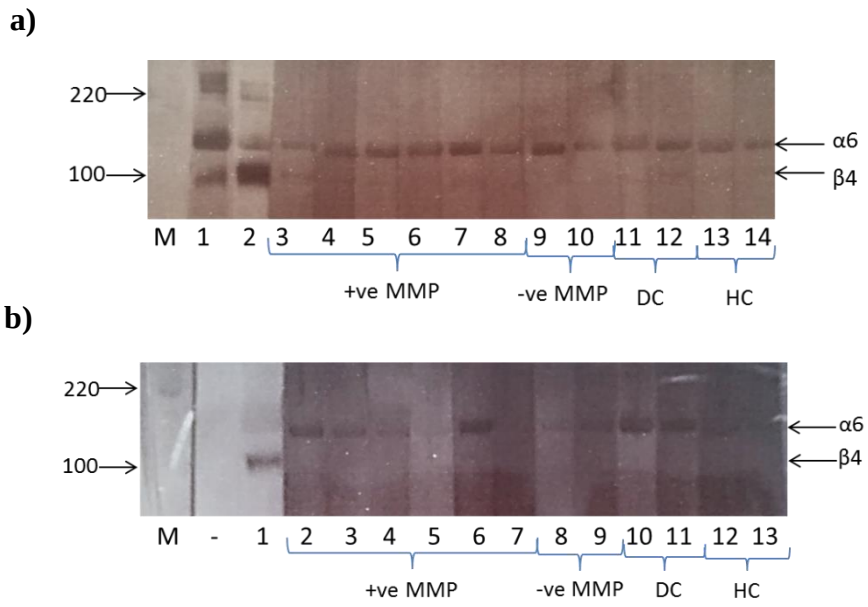


Figure 4-9 Western blots depicting reactivity of serum samples from MMP, disease and healthy controls against the α6β4 integrin α6β4 integrin on a reducing gel (α6 125 Kda β4 100 Kda)**,** M (marker, numbers on the side are the molecular weights), - (no primary antibody), a:1&2 b:1 (probing with anti-α6 / anti-β4 polyclonal antibodies to confirm identity of the separated polypeptides), +ve MMP (MMP serum samples positive to α6β4 on ELISA), -ve MMP (MMP serum samples negative to α6β4 on ELISA), DC (disease controls include PV, LP), HC (healthy control). MMP, DC and HC show bands corresponding to α6 molecular weight (125 Kda) with minimal β4 reactivity (100 Kda). There was no demarcation between the sample groups tested a) A representative western blot showing nonspecific binding to α6 in all tested groups with minimal β4 reactivity b) A representative western blot showing nonspecific binding to α6 in all tested groups with minimal β4 reactivity as well as a negative control that shows no reactivity with the α6 fragment. These representative Western Blots show 16 of the 42 MMP samples analysed

4.5 Discussion

IgG antibodies to α6β4 integrin were detected in 18/100 (18%) of whole saliva samples of MMP patients. This has not been shown before. Together with the previously mentioned results of IgG and IgA antibody detection to BP180-NC16a in saliva (44%), these data help support the notion that saliva provides a suitable diagnostic medium for MMP patients. The detection of salivary antibodies offers an easier medium to diagnose patients as it is painless and easier to acquire.

Serum IgG antibodies were also detected in 36/100 (36%) of samples from MMP patients. The actual prevalence of autoantibodies to the α6β4 integrin in MMP is unclear (Leverkus et al., 2001). Previous studies refer to serum IgG antibodies to α6 integrin or the β4 integrin separately. However, a case report describing the presence of IgG antibodies to both subunits of α6β4 separately in serum of a pure oral MMP patient was published recently (Miyamoto et al., 2014). Therefore, the report possibly represents the first study to detect IgG antibodies directed to both fragments of the α6β4 integrin. Furthermore, the patient showed antibodies to the BP180-NC16a epitope with no reactivity against the C-terminal domain of BP180. These findings further support the heterogeneity of MMP and lack of a clear association between the various clinical sub-types and target antigens. Further studies testing both α6 and β4 integrin fragments separately should be conducted.

The current MMP cohort was then subdivided into pure oral, pure ocular and multisite disease patients and the findings were analysed. Interestingly, the pure oral subgroup showed the highest positivity of IgG antibodies to α6β4 in serum 14/33 (42%), and 7/33 (21%) in whole saliva.

It has been proposed that 100% of untreated oral pemphigoid patients bind exclusively to the α6 fragment on Western blot (Rashid et al., 2006a). However, sera from oral pemphigoid patients' have also been shown to react to both the C-terminal and the NC16a epitopes of BP180, the main target antigen in MMP (Calabresi et al., 2007, Oyama et al., 2006, Carrozzo et al., 2004). More recently, Hayakawa et al reported that 17/30 (57%) of pure oral pemphigoid patients showed IgG antibody to the C-terminal domain of BP180 and laminin 332 (Hayakawa et al., 2014). Therefore, comparative results in the literature vary.

In the multisite disease group, 18/51 (35%) showed IgG antibody reactivity to α6β4 in serum and 11/51 (22%) in whole saliva. Contrary to these findings, it has been

proposed that anti-α6 IgG antibodies are not detected in multisite disease patients but are specific to oral pemphigoid patients (Di Zenzo et al., 2014). However, other studies have reported that multisite disease patients have IgG antibodies directed towards the β4 integrin (Rabelo et al., 2014, Leverkus et al., 2001). Thus there is conflicting data in the literature.

In the pure ocular subgroup, IgG antibody to α6β4 was detected in a quarter of the serum samples from MMP patients but none were detected in whole saliva. Since the results describe reactivity against the whole integrin (α6β4) and not to the separate subunits, a link between a specific target antigen and clinical phenotype has yet to be confirmed. Patients with clinical features of ocular pemphigoid with other site involvement have been reported to show reactivity against BP180 antigen as well (Letko E. et al., 2000). However, in the literature, the β4 integrin is considered by some as the ocular pemphigoid antigen (Tyagi et al., 1996). Antibody titre to the β4 integrin in pure ocular patients has been reported to correlate with disease severity (Letko E. et al., 2000). Leverkus et al described a case of MMP with severe multisite involvement (oral, ocular, laryngeal and genital mucosal involvement) where antibody reactivity was detected to both BP180 and the β4 integrin (Leverkus et al., 2001). Furthermore, he found that the antibody titre to both antigens correlated with disease severity. In addition, it has been reported that a patient with pure ocular pemphigoid showed IgG antibody reactivity to the β4 integrin as well as both IgG and IgA antibody to the C-terminal domain of BP180 (Solano-López et al., 2014). This highlights the ongoing challenge of associating clinical phenotypes with specific antigens.

A possible association between serum and saliva IgG antibodies to α6β4 and oral disease severity scores was looked at. A positive correlation was found between IgG antibody to α6β4 in serum and the oral disease severity score (Spearman r =0.365 p =0.037). Similarly, a positive association was found between IgG antibody to α6β4 in whole saliva and oral disease severity score (Spearman r =0.276 p = 0.0055). In addition, a positive correlation was found between IgG antibodies to α6β4 in both serum and saliva (Spearman r =0.493 p <0.0001) which supports the presence of IgG antibody in whole saliva to α6β4 as a transudate from serum. The extent and severity of disease was correlated with antibody titre to α6β4 in other reports supporting our findings (Rabelo et al., 2014, Sami et al., 2002). In a report about 2 sisters, one with mucosal lesions while the other had predominantly cutaneous involvement, levels of anti-β4 antibodies in serum were detected in the active stage of MMP but not in remission by immunoblot (Rabelo et al., 2014). In addition, Sami et al tested serum samples in 7

patients with severe oral pemphigoid by immunoblot using bovine gingival lysate as a substrate. They found that in patients with active disease the anti-α6 antibody could be detected but not in patients in remission. In this cohort, 39/100 (39%) of the MMP patients with oral disease severity scores < 10 did not show IgG antibodies to α6β4.

IgA antibodies to α6β4 integrin were not detected in sera or whole saliva samples of our MMP patients. Consequently, IgA did not provide a suitable diagnostic biomarker in either serum or whole saliva. There have been no reports of IgA antibody reactivity to α6β4 integrin in MMP patients in either serum or whole saliva in the literature.

No binding was detected to the α6β4 integrin by using BIAcore technology. The observation that anti-α6 and anti-β4 antibodies bound to the immobilised integrin indicates that at least some epitopes remain accessible to fluid-phase antibody following covalent attachment to the sensorchip surface. The absence of any signal on injection of sera from patients over the sensorchip suggests that antibodies are either at very low concentrations or of low affinity.

As the MMP cohort included both pure oral and pure ocular groups, it would have been informative to assess specific target antigen i.e. α6 and β4 separately. However, the Western blot results were inconclusive due to apparent non-specific binding to α6 polypeptide and minimal reactivity with the β4 polypeptide. A commercially available recombinant human α6β4 protein was used, which may account for these unexpected results. The most sensitive method for autoantibody detection against α6β4 suggested is by using α6β4 from bovine gingival lysate (Schmidt and Zillikens, 2013). Furthermore, α6 specific antibodies were detected when using extracts from human gingival lysate (Di Zenzo et al., 2014). Immunoblotting of a hemi-desmosome-rich fraction has also been used to differentiate between the two subunits of the α6β4 integrin (Miyamoto et al., 2014).

With regard to the β4 integrin, previous studies have tested for reactivity to the whole length molecule (205 Kda) or the intracellular domain (140 Kda) but not to the extracellular domain (100 Kda) (Bhol et al., 2003, Bhol et al., 2000). Since the protein used in this study comprises only the extra-cellular domain of each polypeptide, this might explain the minimal reactivity to β4 integrin on Western blots. In contrast, binding to the α6 integrin on Western Blots was noted in all subgroups tested.

4.6 Conclusion

These results showed the presence of IgG antibody to the α6β4 integrin in whole saliva of MMP patients. This has not been previously reported. Furthermore, the IgG antibody levels to the α6β4 integrin in both serum and whole saliva have a significant association with disease severity. Salivary and serum IgG antibodies to the α6β4 integrin were significantly correlated, strongly suggesting that the IgG antibodies in saliva were derived directly from serum, presumably via transudation across inflamed mucosa or via the gingival crevice. These results indicate the suitability of whole saliva in the diagnosis of MMP patients. Further testing, however, is still needed to establish reactivity to the separate integrins i.e. α6 and/or β4.

Although published studies propose a link between β4 and the pure ocular subgroup as well as between α6 and pure oral MMP, the heterogeneity of MMP is clearly evident as numerous studies report autoantibody reactivity to BP180 which is also supported by our results. It would be insightful to establish specific binding results to α6 and /or β4, especially with saliva samples, which may show either dual or single binding to the separate fragments thus providing further clarity to the associations between certain clinical phenotypes and specific target antigens.

4.7 Summary

- Serum IgG antibodies against the α6β4 integrin were detected in 36/100 (36%) of MMP patients
- Salivary IgG antibodies against the α6β4 integrin were detected in 18/100 (18%) of MMP whole saliva samples which has not been previously reported
- Salivary IgG antibody to α6β4 was detected in 21% of both the pure oral and multisite disease groups (7/33 and 11/51) respectively.
- IgA antibodies against the α6β4 integrin were not detected in either serum or saliva
- The highest IgG antibody level detected against α6β4 integrin in serum was in the pure oral subgroup 14/33 (42%), followed by the multisite group 18/51 (35%) and lastly the pure ocular subgroup 4/16 (25%).
- IgG antibodies to α6β4 in both serum and whole saliva were significantly correlated with the oral disease severity score ($p = 0.037$ and $p = 0.0055$) respectively

5 Results PV Study

- **Serum and salivary antibodies to Dsg3 in relation to disease activity (Cross-sectional study)**
- **Sequential antibody titres in PV patients to Dsg3 in relation to therapeutic response (Longitudinal study)**

5 Serum and salivary antibodies in PV to Dsg3 in relation to disease activity and therapeutic response

5.1 Introduction

The target antigen for mucosal lesions is Dsg3 while Dsg1 is the cutaneous antigen (Ding et al., 1997). The mucocutaneous subgroup usually shows IgG autoantibodies to both Dsg1 and 3 antigens. Although a minority of patients have been reported in the literature do not follow the typical clinical phenotype (Jamora et al., 2003).

The antibody titre in serum is well described as reflecting disease activity and has been used to monitor therapeutic response (Daneshpazhooh et al., 2007, Mortazavi et al., 2009). The use of ELISA for testing serum samples for the diagnosis and monitoring of PV patients has been well documented for the past decade (Ishii et al., 1997, Schmidt et al., 2010). ELISA testing is considered more sensitive than indirect immunofluorescence (Ishii et al., 1997).

The presence of IgG antibody in saliva against Dsg3 has been reported in the literature in 3 studies thus far. Anti-Dsg3 IgG antibody has been reported in whole saliva in up to 94% of newly diagnosed PV patients while anti-Dsg1 IgG antibody has been detected in up to 70% of PV patients (Andreadis D, 2006, Hallaji et al., 2010, Hosseein Mortazavi, 2015). Saliva provides a non-invasive and sensitive medium for diagnosing and monitoring diseases. Advances in technology, nanotechnology and proteomics and salivary diagnostics have proven to be highly sensitive in the diagnosis of systemic diseases (Cheng et al., 2014, Krishna Prasad et al., 2013, Hu et al., 2011, Wong, 2006). Salivary biomarkers are currently being used in oral and breast cancer diagnostics with much potential (Arif et al., 2015, Cheng et al., 2014). In this study, the presence of salivary and serum anti-Dsg3 antibodies in PV was investigated.

The aims of this study were to:

1) Establish whether whole saliva might provide a suitable alternative to serum for diagnosing and monitoring PV

2) Investigate whether anti-Dsg3 IgA antibodies could be detected in serum and saliva

3) Establish whether there was an association between serum or saliva anti-Dsg3 antibodies and disease severity

4) Utilize serum and salivary biomarkers in the analysis of disease activity and therapeutic response

5.2 Serum and salivary antibodies in PV to Dsg3 in relation to disease activity (Cross-sectional study)

In this study PV patients' (n=23), healthy controls' (n=17) and disease controls' (MMP n=8, LP n=11) serum, whole and parotid saliva samples were tested against Dsg3 by ELISA. The standard against which all serum and whole saliva samples tested against Dsg 3 is shown in (Fig 5-1 a-b). A summary of patients' demographics, clinical data, immunofluorescence and ELISA results are shown in (Appendix 4). The mean titre of anti-Dsg3 IgG antibodies in both serum (14593 ±22411) and saliva (885 ±1972) from PV patients, was significantly different from those of the controls, both healthy (46.87 ±116.5) and disease (57.07 ±71.12) in all tested parameters using one-way ANOVA ($p < 0.0001$). In addition, the mean titre of serum anti-Dsg3 IgA antibodies (61922 ±56283) were significantly different between the tested subgroups ($p < 0.0003$). All results measured above the mean +2 SD of the healthy controls were considered positive. Control samples were all negative. There was no significant difference in the mean of IgA antibodies to Dsg3 in whole saliva ($p=0.684$) as well as SIgA antibodies to Dsg3 in parotid saliva ($p=0.236$) between the groups tested. Therefore they were not considered diagnostic.

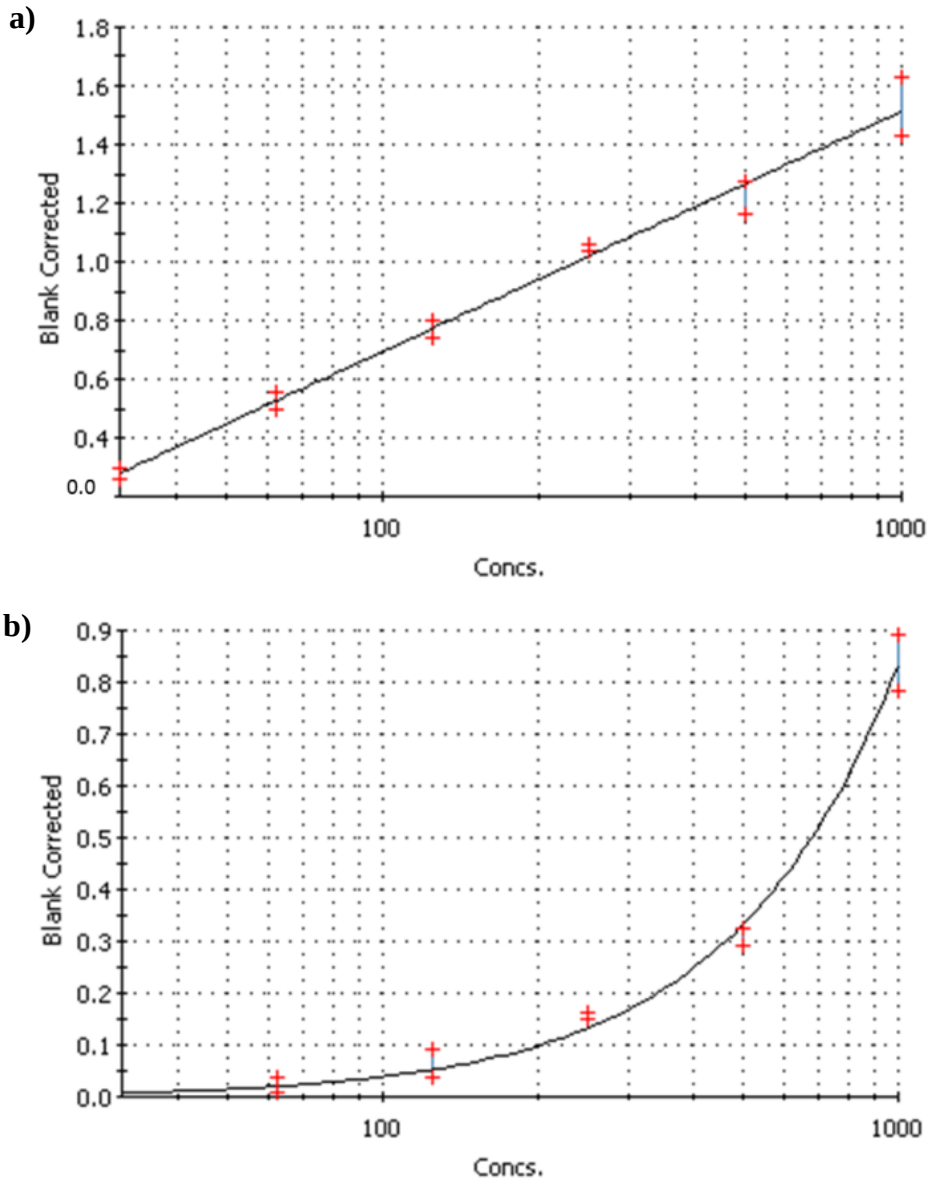


Figure 5-1 Standard curve for serum and whole saliva IgG/IgA antibody testing against Dsg3 by ELISA a) Standard curve for serum and whole saliva IgG antibody testing against Dsg3 by ELISA. b) Standard curve for serum and whole saliva IgA antibody testing against Dsg3 by ELISA. Both figures show a Y-axis which represents blank corrected optical density values and an X-axis representing arbitrary units (0-1000) (100,000 by multiplying by dilution factor 100). The standard curve was constructed using 6 doubling dilutions of a range of pooled antibody positive serum samples. Serum and whole saliva IgG/IgA antibody titres were read in units by calculating the mean of values of four doubling dilutions falling on the reference curve

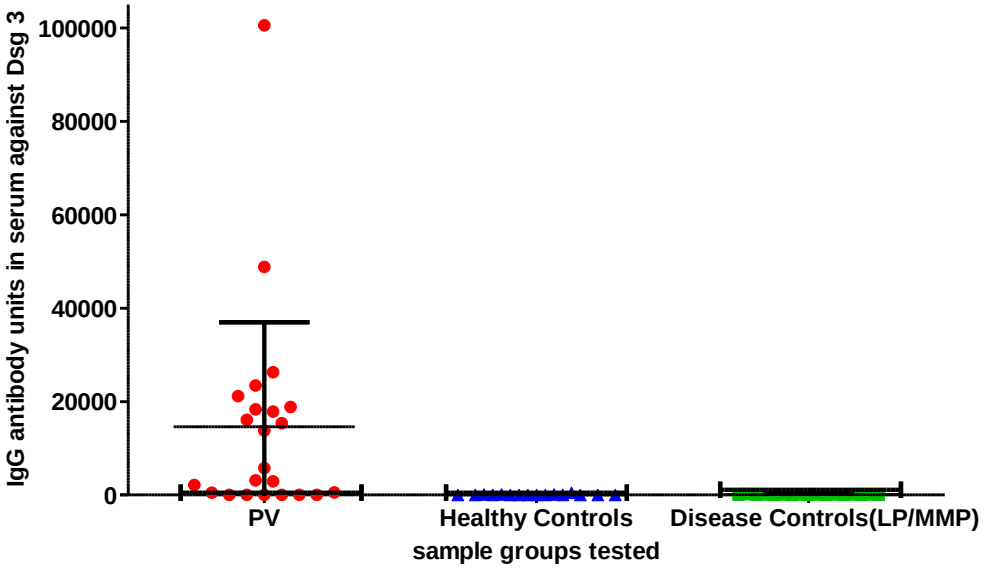
Serum and saliva antibody results to Dsg3:

Serum IgG antibody to Dsg3 was detected in 17/23 (74%) of PV patients. IgA antibody against Dsg3 in serum was detected in 14/23 (61%) of PV patients with a combined positivity (IgG and/or IgA antibody) 18/23 (78%) (Fig 5-2 a-b). Whole saliva IgG antibody to Dsg3 was detected in 14/23 (61%) of PV patients, all of whom were positive for anti-Dsg3 IgG antibody in serum and had active oral disease with an oral disease activity score ranging from (7-57) (Fig 5-2 c). Data summarized in Appendix 4. We were not able to detect reproducible anti-Dsg3 IgA antibodies in whole or parotid saliva (Fig 5-2 d).

Table 5-1 PV Study ELISA results for IgG antibodies against Dsg3 in serum and whole saliva and IgA antibody in serum

PV Study (n=23)	IgG Serum	IgG Whole Saliva	IgA Serum
ELISA Results against Dsg3	**17/23 (74%)**	**14/23 (61%)**	**14/23 (61%)** **Combined (IgG and/ or IgA) 18/23 (78%)**

a)



b)

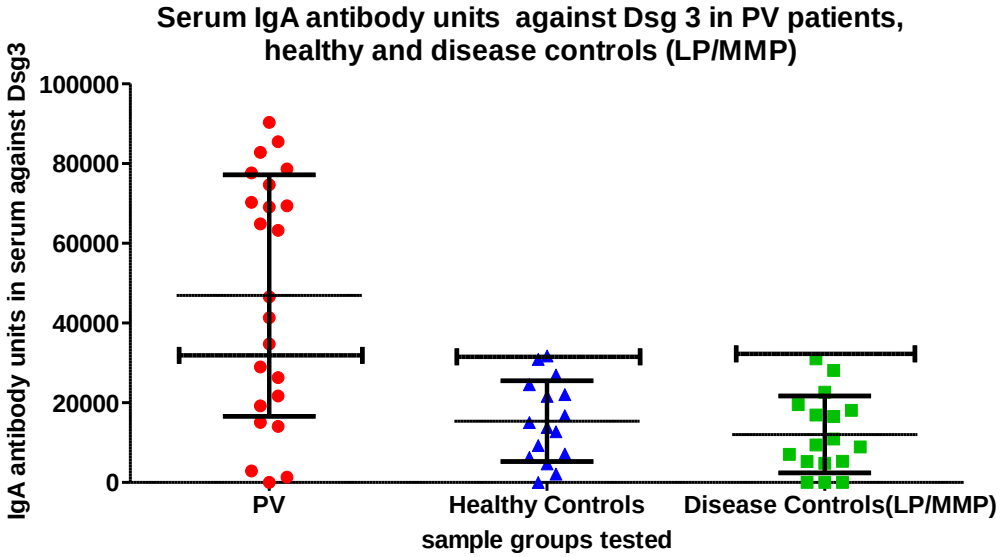


Figure 5-2 IgG and IgA antibodies to Dsg3 ELISA in serum in PV patients, Healthy and Disease controls using ELISA (Mean +/- SD) The Y-axis represents the antibody titre in units while the X-axis shows the sample groups tested. Horizontal I bar indicates cut-of level (mean +2SD of HC). Controls were negative in all. a) Serum IgG antibody units against Dsg3 in PV 17/23 (74%) positive. b) Serum IgA antibody units against Dsg3 in PV 14/23 (61%) positive with a combined result (IgG and/or IgA) of 78% (18/23)

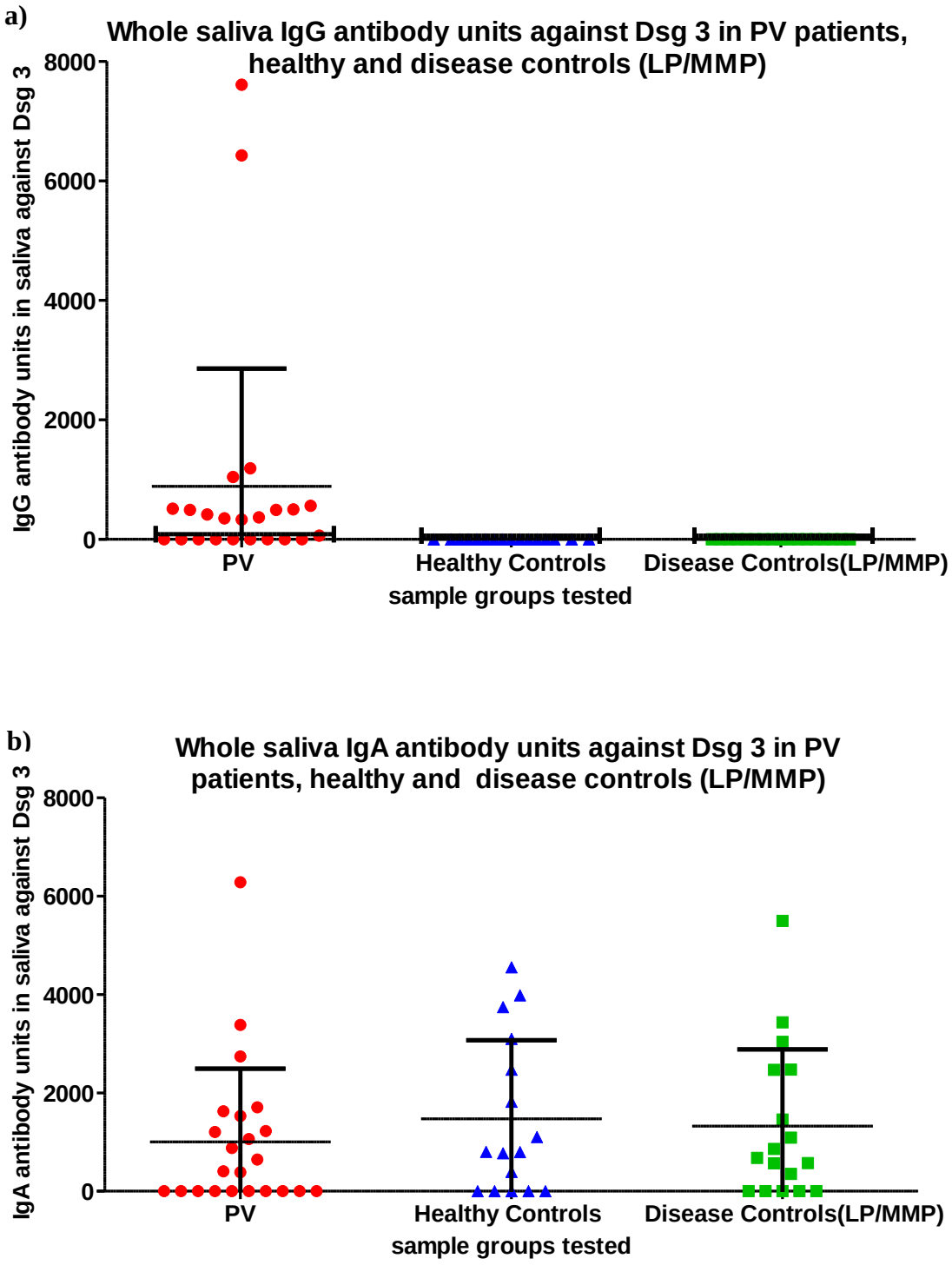


Figure 5-3 IgG and IgA antibodies to Dsg3 ELISA in whole saliva in PV patients, Healthy and Disease controls using ELISA (Mean +/- SD) The Y-axis represents the antibody titre in units while the X-axis shows the sample groups tested. Horizontal I bar indicates cut-of level (mean +2SD of HC). Controls were negative in all. (a) Whole saliva IgG antibody against Dsg3 was positive in 14/23 (61%) PV patients. b) There was no significant difference between the means of the subgroup tested therefore IgA antibody in ws was not considered diagnostic

There was a positive correlation between IgG antibody to Dsg3 in serum and whole saliva of PV patients (Spearman r =0.88 p<0.0001) (Fig 5-4). Patients positive for anti-Dsg3 IgG antibody in saliva were also positive in serum.

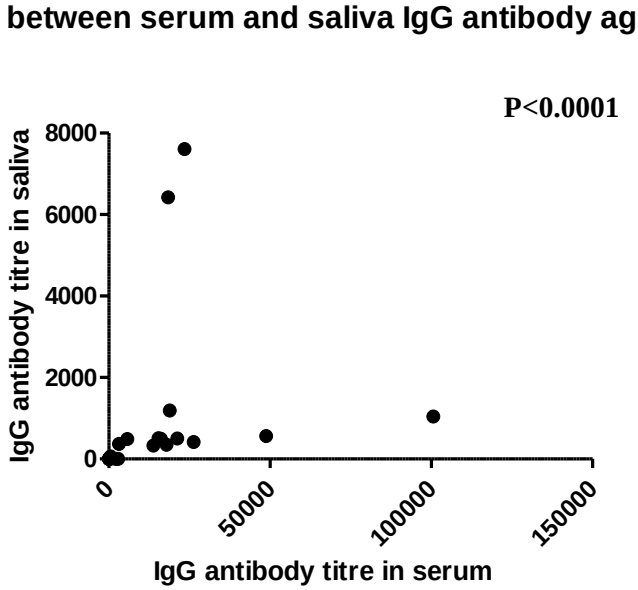


Figure 5-4 Correlation between IgG antibody to Dsg3 in serum and whole saliva Spearman r =0.88 p<0.0001

All 23 PV patients had mucosal lesions while only 8/23 had skin involvement. The patients' sera were also tested for reactivity against Dsg1. Of the eight patients with skin lesions, 3/8 (38%) had positive IgG antibodies to Dsg1 while 5/8 and the remaining 15 mucosal samples were negative. Therefore Dsg1 specific antibodies were not looked at for saliva.

5.3 Sequential antibody titres in PV patients to Dsg3 in relation to therapeutic response (Longitudinal study)

PV patients (n=23) serum and whole saliva, were collected sequentially. Four samples were collected from patients on a 3 monthly basis. The mean severity score was 13.6 (95% c.i. 10.6 to 16.6) and the median was 9.5 (range 0 to 57). The models were validated using the square root transformation of severity given the non-normality detected in this outcome. A significant change in severity was found over time, with the mean severity score decreasing in 2.2 (95% c.i 0.67 to 3.7; p=0.01) for each 3-month block of time. After adjusting for this time effect, only saliva IgG was found to have a

significant effect on severity (mean=0.0025; 95% c.i. 0.0008 to 0.0042; p=0.004). Neither serum IgG (p=0.25) or serum IgA (p=0.08) were found to have any significant effect. A representative sample showing anti-Dsg3 IgG and IgA antibody levels in serum and whole saliva over the collection time period and the oral disease severity scores (Fig 5-5). The anti-Dsg3 antibody titre (IgG serum and whole saliva as well as IgA serum) at the collection time points is demonstrated in (Fig 5-6).

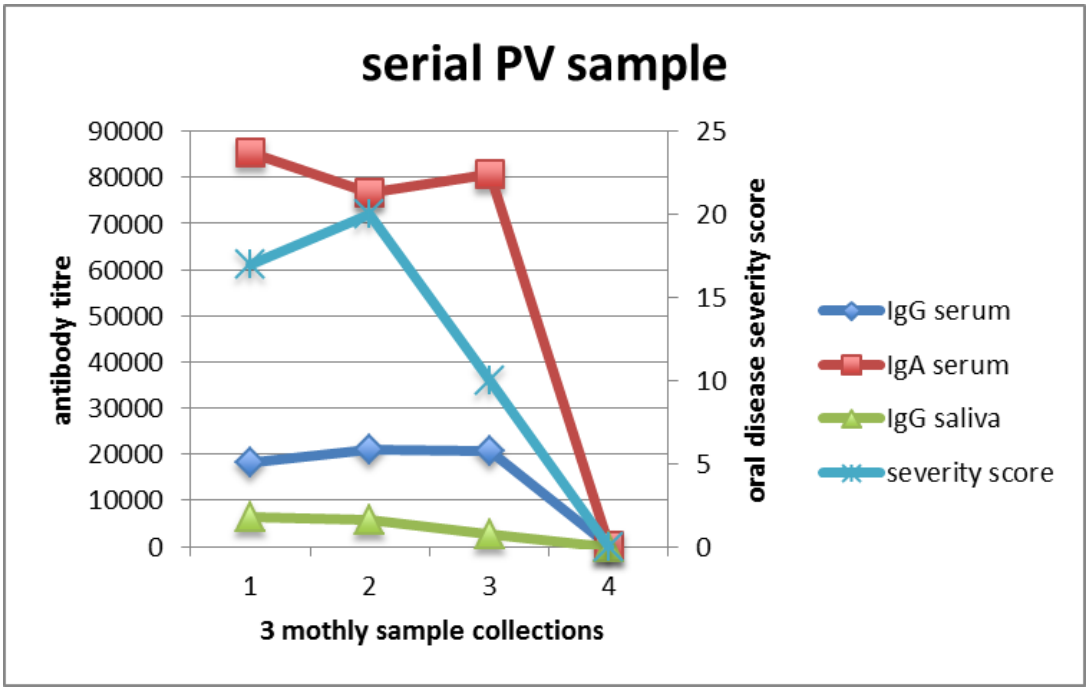
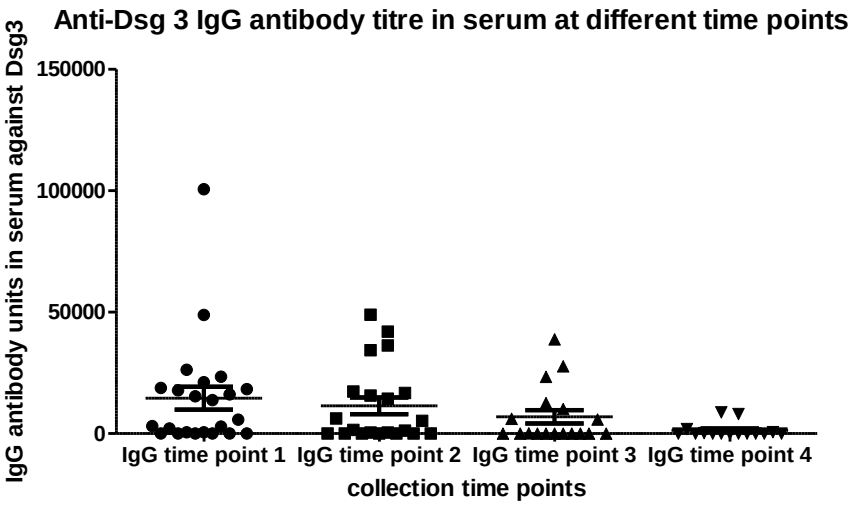


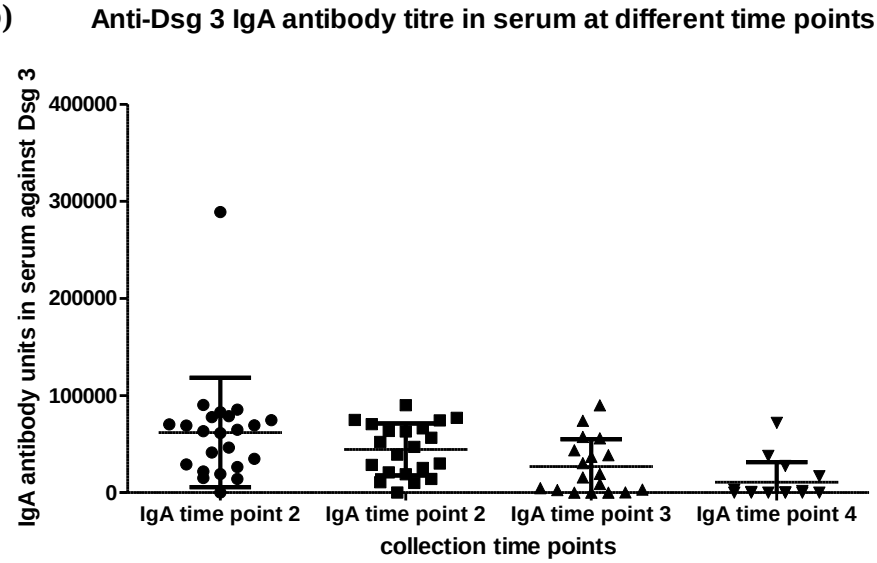
Figure 5-5 Graph showing serial PV sample (1 representative sample) IgG and IgA units to Dsg3 in serum and IgG antibody in whole saliva as well as oral disease severity score

The X-axis represents 4 samples which were collected three monthly. Results of Dsg3 ELISA were plotted: serum anti-Dsg3 IgG and IgA antibodies and whole saliva anti-Dsg3 IgG antibodies, the **left** Y-axis (antibody units). The oral disease severity score, the **right** Y-axis (Oral disease severity score). Red (IgG antibody to Dsg3 in serum), Blue (anti-Dsg3 IgA antibody in serum), Green (IgG antibody to Dsg3 in whole saliva), and Turquoise (oral disease severity score)

a)



b)



c)

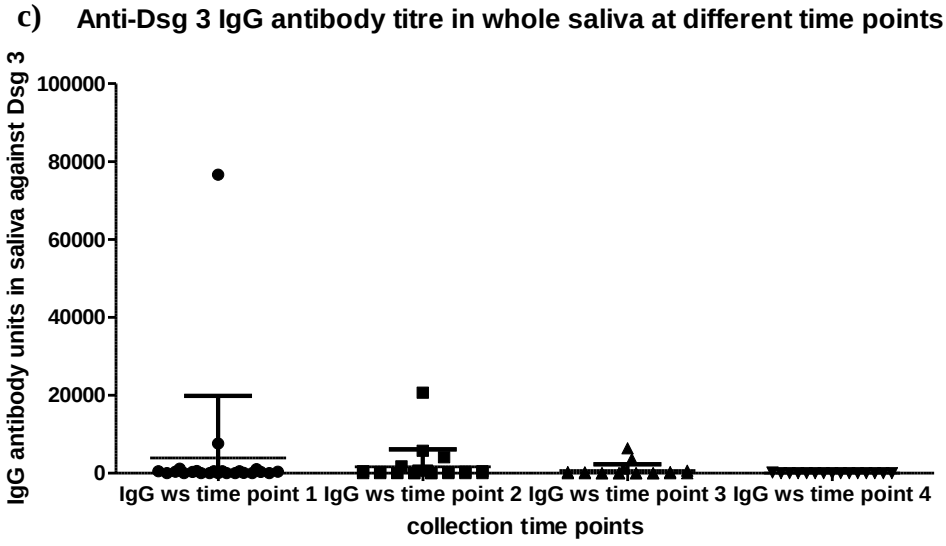


Figure 5-6 Levels of IgG and IgA antibodies to Dsg3 in serum in PV patients at 3-monthly time-points a) anti-Dsg3 IgG antibody level in serum at the different collected time points b) anti-Dsg3 IgA antibody level in serum at the different collected time points c) anti-Dsg3 IgG antibody level in whole saliva at the different collected time points (p=0.004)

5.4 Discussion

The results show that not only does saliva provide a suitable medium for detecting anti-Dsg3 IgG antibodies and the diagnosis of PV, it is also useful in monitoring disease activity. Furthermore, serum IgA antibodies to Dsg3 were detected in sera of PV patients.

Salivary diagnostics have improved with the advancement of technology (Wong, 2006, Pfaffe et al., 2011). Salivary biomarkers are currently being used in oral and breast cancer diagnostics with much potential (Arif et al., 2015, Cheng et al., 2014). Salivary IgG and IgA antibodies to BP180-NC16a in MMP patients were also detected. This further supports the interest in salivary diagnostics for PV.

IgG Anti-Dsg3 autoantibodies:

In this study, both serum and whole saliva samples were tested for autoantibodies targeting Dsg3 antigen. The results showed that 74% (17/23) of the PV patients were positive for IgG antibody to Dsg3 in serum while 61% (14/23) were positive in whole saliva. All patients positive in saliva were also positive in serum for the IgG antibody and a positive correlation between IgG antibodies to Dsg3 in serum and whole saliva was found ($p<0.0001$). These findings concur with three previous reports in the literature describing detection of IgG antibodies to Dsg1 and 3 in whole saliva of PV patients (Hosseein Mortazavi, 2015, Hallaji et al., 2010, Andreadis D, 2006).

In previously reported studies, IgG antibody to Dsg3 in serum has been detected in up to 96% of new PV patients (Andreadis D, 2006, Hallaji et al., 2010, Hosseein Mortazavi, 2015). The patients recruited to these were either new patients or samples were collected during the active stage of the disease. Andreadis tested 27 PV patients and found that 93% had both serum and saliva anti-Dsg3 antibodies while 45% were positive for anti-Dsg1 antibodies in serum and saliva (Andreadis D, 2006). Hallaji et al reported 94% (n=50) positivity in both serum and saliva anti-Dsg3 antibodies. Anti-Dsg1 antibodies were detected in 70% of the PV patients in both serum and saliva (Hallaji et al., 2010). Finally, Mortazavi et al tested 86 PV patients and reported anti-Dsg3 antibodies in 96% of PV sera while 73% showed salivary specific antibodies. Anti-Dsg1 results were positive in 72% of PV patient sera and 36% of saliva (Hosseein Mortazavi, 2015).

The results for serum showed a lower positivity of detection (74%) of IgG antibodies to Dsg3 than these previous studies. This may be explained by the fact that our patients were not new referrals and had been on long term treatment resulting in lower levels of disease activity. This is supported by the observations of significant decreases in serum antibodies over 9 months in sequential samples. The patient cohort was also mainly of the mucosal variant of PV. Only 8/23 had skin involvement of which 3/8 were positive for IgG antibodies to Dsg1 in serum (38%). In contrast, in the mucocutaneous subtype up to 72% had anti-Dsg1 IgG antibody in serum (Hosseein Mortazavi, 2015). Since the majority of our patients had mucosal involvement only, the low positivity to Dsg1 was expected.

Previous studies have reported an association between serum autoantibody to Dsg1 or 3 and therapeutic response (Schmidt et al., 2010). In this study, results did not show a relationship between serum anti-Dsg3 antibody levels and therapeutic response during the study period (in contrast to saliva). This could be due to the small number of patients tested, the short follow up time and again the fact that patients had already been on long term systemic treatment and that the antibody titre had already fallen.

Results for salivary antibodies showed that despite being on long term systemic therapy, IgG antibody to Dsg3 in whole saliva was detected in 61% (14/23) patients in this cohort. This is compared to untreated patients, among whom a positivity of IgG antibodies to Dsg3 has been reported in 70% to 94% of PV patients (Hosseein Mortazavi, 2015, Hallaji et al., 2010). Salivary Dsg1 was not tested for due to the low positivity in serum and lack of cutaneous lesions in this cohort.

IgG antibodies found in whole saliva are predominantly derived from serum. These autoantibodies are transported into saliva by diffusion via the gingival crevicular fluid and breaches in the epithelium. The more inflammation present within the oral cavity, the more leakage of antibodies from serum into whole saliva (Sistig et al., 2002). This forms the basis for testing for IgG antibodies to Dsg3 in whole saliva of PV patients. Since oral lesions are commonly encountered, it would be expected for IgG antibody to be present in saliva during active disease.

The testing of sequential salivary samples has not been previously reported. This study has revealed for the first time that IgG antibodies to Dsg3 in whole saliva are associated with a change in severity score ($p=0.004$). These findings suggest a promising use for salivary anti-Dsg3 detection in whole saliva as part of the monitoring of PV patients. It is interesting that while more patients were positive for IgG in serum overall, this

biomarker was less discriminatory for disease activity. It is therefore important to undertake further studies with a larger cohort and including more patients at the onset of disease.

IgA Anti-Dsg3 autoantibodies:

While the main focus in the literature has been testing for IgG antibodies to Dsg1 and 3 in serum and saliva, we have tested for IgA antibodies to Dsg3 in both serum and saliva. IgA antibody to Dsg3 was detected in serum in 14/23 (61%) of the patients with a combined IgA and/or IgG antibody positivity of 18/23 (78%) patients. None had serum IgA antibodies alone.

On the basis of the presence of Dsg3 IgA antibodies in serum, we would have expected to detect IgA antibodies in whole saliva. However, saliva contains many high molecular weight mucins which sometimes give rise to non-specific binding. Our background activity for IgA antibody was higher than that of IgG antibody, which may have masked the detection of anti-Dsg3 IgA antibody in PV patients. Further work is underway.

IgA positivity alone in PV usually reflects a separate entity labelled as IgA pemphigus (Tajima et al., 2010). However, others have reported that in IgG-driven PV, IgA antibodies to Dsg3 are sometimes detected (Porro et al., 2014). Spaeth et al reported the detection in acute onset and chronic PV of IgA antibody to Dsg3 in 9/15 (60%) and 13/18 (72%) respectively (Spaeth et al., 2001). Similarly, Mentink et al showed serum IgA anti-Dsg3 antibodies in 18/27 (67%) of patients while serum IgA anti-Dsg1 antibodies were positive in 15% (Mentink et al., 2007). Hashimoto et al reported detection of anti-Dsg3 or 1 IgA antibody in 4/22 (18%) of pemphigus patients (Hashimoto et al., 2001). Thus the detection of IgA antibody to Dsg3 in PV patients may be more common than previously assumed (Mentink et al., 2007). It has been proposed that IgA antibody activity to Dsg3 rather than IgG may be associated with the presence of mucosal lesions in PV (Spaeth et al., 2001). Our PV patient cohort showed predominantly mucosal involvement and a high proportion had IgA antibodies.

It is not known whether IgG and IgA antibodies are directed at different epitopes on Dsg3 or whether IgA has a role in disease pathology or epitope spreading. However, Mentink et al found that serum IgA antibody to Dsg3 was elevated **before** serum IgG antibody started to rise (Mentink et al., 2007). Whether IgA antibodies to Dsg3 are

directly involved in the pathogenesis or reflects epitope spreading remains to be elucidated (Tchernev and Orfanos, 2006).

ELISA provides a more sensitive tool for autoantibody detection against the PV target antigens than immunofluorescence (Lenz et al., 1999, Harman et al., 2000c, Heymann, 2009). This also appears to be the case for detecting IgA antibodies in serum to Dsg (Mentink et al., 2007). Whether IgG/IgA pemphigus is a separate entity or whether the typical IgG pemphigus vulgaris has a wider spectrum needs further clarification (Porro et al., 2014). In addition, the detection of IgA antibodies to Dsg3 further supports the hypothesis that PV is a T helper 2 (Th2) autoimmune mediated disease and may suggest a possible pathogenic role for IgA antibodies in PV (Spaeth et al., 2001).

5.5 Conclusion:

This study supports previous reports indicating the value of whole saliva for diagnosis of PV by detecting IgG antibodies to Dsg3. By closely monitoring disease severity and relating this to sequential saliva samples, these results also show that salivary anti-Dsg3 antibodies maybe used in monitoring PV patients. Further studies are needed to more fully explain the role of IgA antibodies to Dsg3 in the pathogenesis and progression of PV.

5.6 Summary:

- Serum IgG antibodies to Dsg3 were positive in 17/23 (74%) of PV patients
- Serum IgA antibodies to Dsg3 were positive in 14/23 (61%) of PV patients. A combined positivity was found in 18/23 (78%) of PV patients.
- Whole saliva IgG antibodies to Dsg3 were detected in 14/23 (61%) of PV patients
- There was a positive correlation between IgG antibody to Dsg3 in serum and whole saliva (Spearman $r = 0.88$ $p < 0.0001$)
- Sequential samples showed that the change in IgG antibodies to Dsg3 in whole saliva had a positive association with the change in oral disease severity scores ($p = 0.004$)

6 General Summary

6 General Summary

The main findings of this thesis are:

Study 1 (a & b): Serum and salivary antibodies to BP180-NC16a in patients with mucous membrane pemphigoid

(a)- In relation to clinical phenotype and disease activity (Cross-sectional study)

(b)- In relation to therapeutic response (Longitudinal study)

- Serum IgG antibodies against the NC16a domain of BP180 were detected in 28/78 (36%) of MMP patients
- Serum IgA antibodies were found in 22/78 (28%). Combined serum IgG and/ or IgA antibodies were positive in 38/78 (48%) patients
- Whole saliva IgG antibodies were detected in 11/78 (14%) of MMP
- Whole saliva IgA antibodies were detected in 30/78 (38%) of MMP patients. Combined results for IgG and/ or IgA antibodies in ws were positive in 34/78 (44%).
- In parotid saliva both secretory IgA and IgA were positive in 9/20 (45%) patients
- There was a positive association between the presence of serum IgG antibodies to BP180-NC16a and multisite disease lesions ($X^2 = 11.9$ p =0.003)
- A positive association was found between the presence of IgA antibody to BP180-NC16a in whole saliva and the presence of combined oral and ocular disease ($X^2 = 6.23$ p =0.0156)
- Pure ocular MMP patients showed no IgG antibody reactivity to BP180-NC16a in either serum or saliva but a higher level of IgA antibody to BP180-NC16a in serum and saliva (38% and 44% respectively) than other clinical subgroups
- The greater the number of involved sites involved in multisite disease MMP, the higher the IgG antibody titre in serum and whole saliva against BP180-NC16a (p =0.017 and p =0.019, respectively)
- Sequential samples showed that the change in serum antibodies (IgG and IgA) was associated with a change in oral disease severity scores (p =0.048 and 0.033, respectively) following therapeutic response

Study 2: Serum and salivary antibodies to alpha 6 beta 4 integrin in relation to clinical phenotype and disease activity (Cross-sectional study)

- Serum IgG antibodies against the α6β4 integrin were detected in 36/100 (36%) of MMP patients
- IgG antibodies to the α6β4 integrin were detected in 18/100 (18%) of MMP whole saliva samples
- IgA antibodies against the α6β4 integrin were not detected in either serum or saliva
- The highest IgG antibody level detected against the α6β4 integrin in serum was in the pure oral subgroup 14/33 (42%), followed by the multisite group 18/51 (35%) and lastly the pure ocular subgroup 4/16 (25%)
- IgG antibody to α6β4 in whole saliva, was detected in 21% and 22% of both the pure oral and multisite disease (7/33 and 11/51, respectively)
- IgG antibodies to α6β4 in both serum and whole saliva were significantly correlated with the oral disease severity score was ($p = 0.037$ and $p = 0.0055$, respectively)

PV study: Serum and salivary antibodies to Dsg3 in relation to disease activity (Cross-sectional) and sequential antibody titres in PV patients to Dsg3 in relation to therapeutic response (Longitudinal study)

- Serum IgG antibodies to Dsg3 were positive in17/23 (74%) of PV patients
- Serum IgA antibodies to Dsg3 were positive in 14/23 (61%) of PV patients. A combined positivity (IgG and/or IgA) was found in 18/23 (78%) of PV patients
- Whole saliva IgG antibodies to Dsg3 were detected in 14/23 (61%) of PV patients
- There was a positive correlation between IgG antibody to Dsg3 in serum and whole saliva (Spearman $r = 0.88$ $p < 0.0001$)
- Sequential samples show that the change in IgG antibodies in whole saliva had a significant association with the change in severity scores ($p = 0.004$)

In this thesis, whether IgG and IgA antibodies to two target antigens, the NC16a domain of BP180 and to α6β4 integrin, in MMP and to the Dsg3 antigen in PV could be detected by ELISA in both serum and saliva was investigated. Moreover, whether these antibodies could be used in the assessment of disease severity and therapeutic response was looked at. In addition, the thesis aimed to identify whether certain clinical phenotypes were related to specific target antigens in MMP.

A). MMP

Serum and salivary autoantibodies:

-BP180-NC16a

In this cohort, 36% had circulating IgG antibodies in serum directed against the NC16a domain of BP180. Serum IgA antibodies to BP180-NC16a in serum were detected in 28% of the patients with a combined positivity of 48%. Similarly, immunofluorescence results from this study show that 35% of the patients were positive on IIF. Although our results may seem to be comparatively low, this may in part be due to the majority of patients being on long-term treatment and thus having lower levels of disease activity as has been shown elsewhere (Sami et al., 2002). In addition, the higher positivity with IIF reported in the literature corresponds to reactivity against any of the BMZ components not to a single antigen (Setterfield J. et al., 1998, Oyama et al., 2006).

Of particular interest, the data has shown that whole saliva samples were reactive to the NC16a domain of BP180 with IgG antibodies in 14% of patients, IgA antibodies in 38% and overall in 44% of the patients. Salivary antibodies to BMZ antigens have not been shown before in MMP. There has been one previous study reporting that neither serum nor salivary autoantibodies were detected against BP18-NC16a in MMP patients (Andreadis D, 2006). The low percentage positivity in whole saliva may reflect the fact that the majority of patients had well controlled disease at the time of sampling. Only 50% of the patients had a positive clinical score for oral disease, most of whom were mildly erythematous rather than having active ulceration.

Specific antibody reactivity to the C terminal domain of BP180 on ELISA was not shown due to the high reactivity of serum to GST on ELISA plates. Therefore antibody reactivity to different epitopes of BP180 was not demonstrated.

-α6β4:

MMP serum and salivary samples were tested for reactivity with the α6β4 integrin. The results have shown that 36% and 18% of MMP patients were positive for IgG antibodies to the α6β4 integrin in serum and whole saliva respectively. Salivary antibodies to the α6β4 integrin in MMP have not been reported previously. Western blot testing of the separate α6 and β4 integrins were not conclusive. Non-specific binding in the western blots resulted in the inability to demarcate between the MMP positive or negative sera and between the control groups. BIAcore results were also

inconclusive as reactivity to the reference cells could not be differentiated. Therefore reactivity to the separate integrins was not shown.

Mucosal antibodies:

Parotid samples were tested to attempt to differentiate between IgA present as a serum transudate (detected as a monomer), from local production (secretion from the gland and detected as dimeric IgA and secretory component positive). Interestingly, 45% of parotid samples were both IgA and secretory component positive. These results have been demonstrated for the first time in saliva of these patients. Size exclusion chromatography showed that secretory component (which has a molecular mass of 80 Kda) is associated with a high molecular weight complex (>250 Kda), presumably dimeric SIgA thus confirming the presence of locally produced antibodies.

Although the precise mechanism of how these antibodies are stimulated has not been ascertained. It is possible to speculate about a mucosal immune response. Whether these antibodies are produced due to damage occurring from a distant mucosal site involvement (e.g. ocular mucosa), or whether subclinical damage within the salivary gland occurs exposing BP180 in their basement membrane has yet to be confirmed (Gonzalez et al., 2011, MacPherson et al., 2008).

Clinical phenotype (Pure oral, pure ocular and multisite disease):

-BP180-NC16a:

The MMP cohort was further divided into pure oral, pure ocular and multisite disease clinical subsets. A positive correlation was found between the number of sites involved in multisite disease and the level of IgG antibodies to BP180-NC16a in both serum and saliva ($p=0.017$ and 0.019 respectively). Interestingly, no IgG antibody was detected in the pure ocular phenotype in both serum and whole saliva. They did however, have the highest levels of IgA antibody to BP180-NC16a in serum and saliva (38% and 44% respectively). Our results suggest a possible link between pure ocular MMP and IgA reactivity to BP180-NC16a. A recent study suggests an association between the ocular subgroup and IgA antibody in serum targeting the C-terminal domain of BP180 (Solano-López et al., 2014).

-α6β4:

By analysing the reactivity in subsets in this cohort of patients to α6β4 integrin, the pure oral subgroup showed the highest level of IgG antibodies in serum to α6β4 at 42%. On the other hand, the multisite subgroup had the highest level of anti- α6β4 IgG antibodies

in whole saliva at 22%. The pure ocular group were positive for IgG antibody to α6β4 in serum but not in saliva. In order to better appreciate these results, testing the samples against the separate fragments α6 and β4 is essential. Consequently, associations between distinct clinical phenotypes and specific target antigens and epitopes were speculated on but not confirmed.

Disease severity and therapeutic response:

- A positive correlation was found between the presence of serum IgG antibodies against BP180-NC16a and the presence of multisite disease lesions ($p=0.003$)
- A positive association was found between the presence of salivary IgA antibodies to B180-NC16a and the presence of oral and ocular lesions in MMP patients ($p<0.02$)
- The greater the number of sites involved, the higher the IgG antibody level to BP180-NC16a in serum and whole saliva detected ($p<0.02$ for both)
- A positive correlation was found between IgG antibodies to BP180-NC16a in whole saliva and disease severity in multisite disease patients ($p=0.022$)
- Serum and salivary IgG antibodies to α6β4 integrin had a positive correlation with oral disease severity score ($p=0.0370$ and $p=0.0055$ respectively)

IIF titres are usually used to monitor disease severity in MMP patients. Sequential samples showed a positive association between the change in serum antibodies to BP180-NC16a (both IgG and IgA) and the change in severity score ($p=0.048$ and $p=0.033$ respectively). These results suggest the valuable use of ELISA in monitoring MMP patients clinically over the course of their treatment.

B). PV

Serum and salivary autoantibodies:

-Anti-Dsg3 IgG antibody:

Regarding PV, 74% of the cohort had IgG antibodies detected in serum against Dsg3 and 61% in saliva. A positive correlation was found between serum and salivary anti-Dsg3 antibodies ($p<0.0001$). Other publications have reported the detection of anti-Dsg3 IgG antibody in whole saliva (Hosseein Mortazavi, 2015, Hallaji et al., 2010, Andreadis D, 2006).

-Anti-Dsg 3 IgA autoantibody:

Interestingly, 61% were positive for IgA antibody to Dsg3 in serum (yielding a combined positivity of IgG and/or IgA of 78%). Generally, IgA positivity is considered

as a separate entity; IgA pemphigus. However, there are publications in the literature in which IgA antibody was detected in serum of PV patients targeting Dsg3 (Spaeth et al., 2001, Mentink et al., 2007, Hashimoto et al., 2001). Whether IgG/IgA pemphigus is a separate entity or whether typical IgG pemphigus vulgaris has a wider spectrum needs further clarification (Porro et al., 2014).

Disease severity and therapeutic response:

The sequential results showed that IgG antibody to Dsg3 in saliva had a significant correlation with the change in severity score ($p=0.004$). This has not been previously described as earlier reports only established the detection of anti-Dsg3 antibodies in saliva. These results indicate the usefulness of saliva not only in diagnosing the disease, but monitoring it as well. This was not shown for serum in this cohort which could be due to the small number of samples tested and a shorter follow up time.

The results confirm what has been previously shown regarding Dsg3 serum and saliva IgG antibodies being both sensitive and specific for diagnosing if PV. In addition, it has been shown that salivary anti-Dsg3 antibodies are useful in monitoring PV patients. The level of Dsg3 salivary IgG antibodies has a significant relation with therapeutic response in PV. Anti-Dsg3 IgA antibodies are detected in serum of PV patients. Further research is required to better understand the significance of this in the pathogenesis of the disease.

BP180-NC16a serum IgG and IgA antibodies both had a positive correlation with therapeutic response in MMP. Salivary biomarkers, IgG antibodies to BP180-NC16a and α6β4 may be useful in the diagnosis of MMP patients as well as salivary IgA antibodies to BP180-NC16a. The novel finding of locally produced antibodies may provide further insight into the pathogenesis of the disease which may explain the propensity of oral lesions**.** These new findings demonstrate the value of saliva as an alternative to serum in the diagnosis of MMP as well as a being a valuable medium for furthering the understanding of disease pathogenesis.

Conclusions and Future Work

7 Conclusions and Future Work

7.1 Conclusions

The cohort of patients was recruited included predominantly mucosal PV and the full spectrum of MMP; i.e. pure oral, pure ocular and multi-site disease patients. The assessment of disease severity and sequential activity was made using a previously published scoring methodology initially published for use in oral LP (Escudier et al., 2007). As both MMP and PV are rare, most patients recruited were already on long term treatment and follow up which may, in part, have resulted in the lower levels of disease activity and of serum and salivary antibody activity. This was considered as an unavoidable limitation of the study.

The main conclusions drawn regarding each aim were as follows:

1. To develop a sensitive salivary assay to diagnose and monitor MMP and PV:
    a. MMP: Salivary antibodies (both IgG and IgA) to BP180-NC16a could be detected in both whole and parotid saliva of MMP patients. In whole saliva, the combined positivity (both IgG and IgA) was 44% which was similar to that of serum at 48%. Furthermore, the novel finding of SIgA antibodies to BP180-NC16a were detected in both whole and parotid saliva of MMP patients. The fact that some patients were found to be a) antibody negative in serum but had positive parotid samples for SIgA anti-BP180-NC16a antibodies, b) that parotid antibodies detected by anti-SC or by anti-IgA showed a strong correlation and c) antibody migrated at a molecular weight of dimeric IgA in chromatography, strongly suggests that these antibodies were mucosally derived. This finding may help explain the propensity for oral lesions in MMP.

Salivary antibodies against the α6β4 integrin were detected and reported for the first time. IgG antibodies targeting α6β4 were detected in whole saliva (18%) of MMP patients but no IgA reactivity was detected. The results overall show the importance of saliva as a medium to be tested in the diagnosis and monitoring of MMP patients. Since antibodies can be detected in serum (36%), whole saliva (18%) or both (36%), combined testing of serum and saliva to multiple antigens should therefore provide more sensitive results. This is further supported by the lack of IgG reactivity to BP180 in the pure ocular group. This group is generally known to be more serum antibody

negative therefore combined testing may offer a more sensitive method for antibody detection especially for IgA.

b. PV: IgG antibodies targeting Dsg3 were detected in whole saliva of PV patients and in serum. A positive correlation was found between serum and salivary anti-Dsg3 antibodies ($p<0.0001$). Although the percentage of antibody positive patients was not as high as those reported in the literature, the fact that the majority of patients were on long term treatment may be responsible for the low levels of disease activity. Interestingly, serum IgA antibodies targeting Dsg3 were detected in 61% of PV patients with a combined positivity of 78%. IgA antibody detection to Dsg3 in IgG driven PV has been reported previously. How anti-Dsg3 IgA affects the pathogenesis has yet to be elucidated.

Therefore, in both MMP and PV, a sensitive salivary assay was developed to help diagnose and monitor both diseases. This can be very helpful for patients with severe blistering in which obtaining a serum sample could be difficult. It would be economic as training is not required for the sample collection and collection of saliva samples is pain free, less time consuming and better accepted by the patients. It would also facilitate screening large numbers of patients for population studies. There are numerous applications in which salivary testing could be promoted as specific antibodies targeting certain antigens can be detected.

2. To utilize serum and salivary biomarkers in the analysis of disease severity and therapeutic response:

a. MMP: A positive correlation was found between the presence of serum IgG antibodies against BP180-NC16a and the presence of multisite disease lesions ($p=0.003$). A positive association was found between the presence of salivary IgA antibodies to B180-NC16a and the presence of oral and ocular lesions in MMP patients ($p<0.02$). Notably, the higher the number of sites involved, the higher the IgG antibody level to BP180-NC16a in serum and whole saliva detected ($p<0.02$ for both). A positive correlation was found between IgG antibodies to BP180-NC16a in whole saliva and disease severity in multisite disease patients ($p=0.022$). In addition, serum and salivary IgG antibodies to $\alpha6\beta4$ integrin had a positive correlation with severity score ($p=0.037$ and $p=0.0055$ respectively). These results suggest the valuable use of ELISA, testing both serum and saliva, in monitoring the

patients' clinically over the course of their treatment as oral severity scores and the level of antibody detection correlate.

Sequential samples showed that the change in serum antibody titre to BP180-NC16a (both IgG and IgA) had a significant relationship with the change in severity score ($p=0.048$ and $p=0.033$ respectively). This indicates that the level of antibodies to BP180-NC16a in serum follows the therapeutic response.

b. PV: The role of IgA antibody to Dsg3 in the pathogenesis of PV has yet to be fully explained. It is therefore, of much interest to further study this cohort of patients with both IgG and IgA antibodies to Dsg3 in an attempt to better understand the significance of IgA antibody in the disease progression.

Sequential samples showed that the change in IgG antibody level to Dsg3 in whole saliva significantly related to the change in severity score ($p=0.004$). This means that the level of antibody reflected the therapeutic response. Although others have reported the detection of IgG antibody to Dsg3 in whole saliva, the potential use of whole saliva in monitoring patients' disease progression has not been described. Serum samples did not show the same relation in this cohort. This could be due to the small number of samples tested and a shorter follow up time which was a limitation of this study in addition to patients already having long term treatment.

Therefore, in both MMP and PV, serum and salivary biomarkers proved important in the analysis of disease severity and therapeutic response. The potential for saliva as a medium for biomarker detection is increasingly being noticed. Not only is it effective in the diagnosis, but it also shows potential for disease monitoring as well.

3. To investigate whether distinct clinical phenotypes are associated with specific target antigens and epitopes in MMP:

MMP is a heterogeneous disease. Although clinical phenotypes have been described; pure oral, pure ocular and multisite disease, a definitive relationship between a certain clinical subtype and target antigens or epitopes has yet to be ascertained. Previous associations between α6 and the pure oral subgroup as well as β4 and the pure ocular subtype have been proposed. The cohorts' samples were tested for reactivity against BP180-NC16a and the α6β4 integrin.

Since a demarcation between MMP and controls was not shown when testing the GST fusion proteins (NC16a and 4574) by ELISA, reactivity to the C-terminal domain of BP180 was not established. This was considered as a limitation of the study. The

results show that the pure ocular subgroups had no IgG antibody reactivity to BP180-NC16a in either serum or whole saliva. They did however, have the highest level of specific IgA antibody directed to BP180-NC16a in both serum and saliva. This denotes a distinct group in which IgA antibody reactivity to BP180-NC16a is detected with no IgG antibody. As previously mentioned, this supports the importance of combining both serum and salivary testing for diagnosing and monitoring the patients. The multisite disease patients had the highest level of IgG antibody against BP180-NC16a in serum and whole saliva. This does not come as a surprise since patients with multisite involvement generally have a more active disease and BP180 is the main target antigen in multisite disease patients. Consequently, it is still unclear whether clinical phenotypes are therefore related to specific epitopes of BP180.

The α6β4 integrin data shows that IgG antibody to α6β4 in serum was most abundant in the pure oral subgroup. The high reactivity in the pure oral subgroup to α6β4 only highlights the importance of this antigen with regards to this clinical phenotype. IgG antibody to α6β4 in whole saliva was detected at a higher level in the multisite disease group. This supports the previous findings regarding the higher level of IgG detected in saliva in multisite disease patients especially as these patients are more likely to have higher serum IgG antibody levels to BP180-NC16a. Reports in the literature mainly test the separate fragments as opposed to the full α6β4 integrin. When testing of the separate fragments α6 and β4 by westerns blots, results showed nonspecific binding to α6 with minimal β4 reactivity. Therefore, results of separate integrin reactivity were not established which was a limitation of this study. Consequently, associations between distinct clinical phenotypes and specific target antigens and epitopes were speculated on but not confirmed.

Finally:

- Since antibodies to BP180-NC16a and α6β4 integrin can be detected in serum, whole saliva or both, combined testing of both serum and whole saliva should therefore, provide more sensitive results.
- The detection of mucosally derived antibody requires further investigation in a larger cohort including patients with untreated disease and using parallel mucosal secretions e.g. tears will be most informative.
- To further increase the sensitivity of saliva as a diagnostic and disease monitoring medium, the additional testing for reactivity with further epitopes on BP180, laminin 332, α6 and β4 integrin separately is likely to be most

informative. The additional screening of these antigens may broaden the value of ELISA and may correlate more closely with disease phenotypes.

- The novel findings have shown that not only are NC16a-specific salivary IgG and IgA antibodies present in MMP patients but that this medium may have a greater sensitivity for detecting IgA autoantibodies than serum.
- The results support previous reports in the literature indicating the value of whole saliva for diagnosing PV by detecting IgG antibodies to Dsg3. By closely monitoring disease severity the results also show the use of salivary anti-Dsg3 antibodies in monitoring PV patients.
- Further studies are needed to more fully explain the role of IgA antibodies to Dsg 3 in the pathogenesis and progression of PV.

7.2 Future work

The results of this thesis give rise to further questions that need investigation in both MMP and PV:

- The demonstration of SIgA antibodies for the first time suggests that testing of tears and vaginal washes for SIgA antibodies might elucidate the involvement of mucosal immune responses in MMP.
- Biopsy of minor salivary glands and immunofluorescence testing should be considered to determine if there is direct involvement of salivary glands as targets in MMP. In addition, collecting saliva from minor salivary glands in order to test for SIgA in MMP patients to determine if there is local oral production of SIgA antibodies.
- Testing saliva samples against different target antigens (e.g. Laminin 332) for specific SIgA antibody detection would provide further insight to the extent of mucosal immunity involvement in MMP and antigens targeted.
- Testing further antigens and immunoglobulin isotypes (IgG, IgA); laminin 332, C-terminal domain of BP180 and the separate α6 and β4 integrin fragment by Luminex or ELISA to investigate whether an association between specific clinical phenotypes and target antigens or epitopes exists in MMP.
- Testing the total IgG fraction of MMP patients' sera to the separate α6 and β4 integrin fragments in Western blot for more specific results of antigen reactivity.

- MMP sequential samples to be tested for reactivity against the different antigens for both serum and saliva to gauge biomarkers for disease severity and therapeutic response.
- Cytokine testing (IL-4, 5, 6, 8 TGB-β) which may elucidate the scarring capacity and in sequential samples whether they provide prognostic biomarkers for disease activity and scarring potential.
- Immunoglobulin subclasses IgG1-4 and IgA1 and 2 and their role in pathogenesis. In addition, testing for IgE and IgM antibody reactivity in serum and saliva to investigate their role in pathogenesis.
- The role of IgA antibody in the pathogenesis of PV needs to be investigated. This could be established by mimicking animal model studies used in describing the role of IgG antibody in PV.
- Testing PV sequential samples (serum and saliva) for anti-Dsg1 and 3 IgG and IgA antibodies in cutaneous and mucocutaneous PV to investigate disease severity and therapeutic response.
- Avidity of antibodies, especially in relation to clinical phenotypes, to determine if the avidity of autoantibodies in an antibody mediated disease is related to disease activity.
- Larger numbers of patients with MMP so that oral phenotypes can be more readily defined and analysed in respect of target antigens and antibody avidity.

8 Appendices

8 Appendix

Appendix 1 Ethical Approval

East Central London REC 1

Royal Free Hospital
Pond Street
London
NW3 2QG

Tel: 020 7794 0552

Mr John Dart
Consultant Ophthalmologist
Moorfields Eye Hospital
162 City Road
London, EC1V 2PD

14 May 2010

Dear Mr Dart

Study title:	**Autoantigen and genetic determinants of disease in mucous membrane pemphigoid**
REC reference:	**09/H0721/54**
Amendment number:	**1**
Amendment date:	**24 April 2010**

The above amendment to include paired saliva and serum samples and to collect samples from race and age-matched controls, was reviewed on 14 May 2010 by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

NHS
Health Research Authority

NRES Committee London - Westminster
Research Health Authority
Ground Floor, Skipton House
80 London Road
SE1 6LH
Telephone:
Facsimile

22 November 2012

Dr Jane Setterfield
Reader/Honorary Consultant in Dermatology in relation to Oral Disease
King's College London
Floor 23 Department of Oral Medicine
Guy's Hospital Campus
London
SE1 9RT

Study title: **Novel Biomarkers in the Diagnosis and Pathogenesis of Immunobullous Disorders**

REC reference: **12/LO/1350**

Dear Dr Setterfield

Thank you for your letter of 04 October 2012, responding to the Committee"s request for further information on the above research and submitting revised documentation.

The further information was considered [in correspondence] by a sub-committee of the REC. A list of the sub-committee members is attached.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Appendix 2 Patient Consent

Department of Oral Medicine

Floor 22 Tower Wing

Tel: 020 7188 4399

Fax: 020 7188 4401

Review Appointments: 020 7188 4343

New Appointments: 020 7188 1772

Clinic: Floor 22 Tower Wing

www.kcl.ac.uk/depsta/dentistry

www.guysandstthomas.nhs.uk/services/ambulatory/dental/oralmedicine.aspx

Guy's Hospital

Great Maze Pond

London SE1 9RT

Main switchboard: 020 7188 7188

Professor S J Challacombe PhD FDSRCS FRCPath FMedSci

Dr J F Setterfield BDS MBBS DCH DRCOG MD FRCP

Pt ID for this Study:

CONSENT FORM for patients

Title of Project: Novel Biomarkers in the Diagnosis and Pathogenesis of Immunobullous Disorders (REC ref: 12/LO/1350)

Researchers: Dr Jane Setterfield, Professor S Challacombe, Dr S Ali

	Tick box
1. I confirm that I have read and understand the information leaflet (Version 2 dated 04.10.12) for the above study. I have had the opportunity to consider the information and ask questions.	☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	☐
3. I understand that my donated blood, saliva and tissue samples will be stored and studied in laboratories of the Department of Clinical and Diagnostic Sciences at Guy's Hospital and that these may be used for future, ethically approved studies. I give permission for this.	☐
4. I understand that my serum and saliva samples previously stored	

within the Oral and Diagnostic Sciences Department at Guy's Hospital may be used in this study. I also understand that these may be used in future ethically approved studies. I give my permission for this. ☐

5 I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by researchers at Guy's Hospital and the regulatory authorities or from the NHS Trust, where it is relevant to my participation in the study. I give permission for access to my records. ☐

6. I agree that my GP and dentist can be informed of my participation in this study. ☐

7. I agree to take part in this study. ☐

Name of Patient	Signature	Date

Name of Physician	Signature	

Appendix 3 Patient demographic, clinical data and ELISA results

MMP Samples	Sex / race	Age	Duration since diagnosis	Pure Oral	Pure Ocular	Multisite Disease	DIF	IIF	ODSS	IgG S		IgA S BP180-NC16a	IgG ws ELISA		IgA ws BP180-NC16a	SC P (BP180-NC16a)	IgA P (BP180-NC16a)	WB Laminin 332/4575
										BP180-NC16a	α6β4		BP180-NC16a	α6β4				
1	f/c	67	10 yrs		y		pos	pos	10	neg	neg	neg	neg	neg	neg			-/-
2	m/c	65	19 yrs		y		pos	NA	0	neg	neg	neg	neg	neg	neg			
3	m/c	62	15 yrs		y		neg	neg	0	neg	neg	neg	neg	neg	neg			-/-
4	m/c	67	9 yrs		y		neg	neg	0	neg	neg	neg	neg	neg	*pos*			-/-
5	f/c	58	11 yrs			or/C,oc	pos	pos	26	neg	neg	neg	neg	neg	neg			+/-
6	m/c	81	9 yrs			or/C,oc,gen	neg	NA	21	pos	neg	pos	neg	neg	Pos∨			
7	f/c	72	12 yrs			or/G,oc	neg	pos	8	pos	neg	neg	neg	neg	neg			-/-
8	f/c	65	7 yrs			or/G,oc	pos	pos	17	pos	pos	neg	neg	pos	neg			
9	m/a	47	5 yrs			or/C,sk,oc,n-ph	pos	pos	42	pos	neg	neg	neg	neg	neg			
10	f/c	76	8 yrs			or/G,n-ph	pos	pos	12	neg	neg	neg	neg	neg	*pos*			+/-
11	f/c	75	7 yrs			or,sk,n-ph (inactive no oral lesions)	pos	neg	2	pos	neg	pos	neg	neg	neg			-/-
12	m/c	72	12 yrs			or/C,sk,oc,n-ph	pos	pos	26	pos	neg	neg	neg	neg	neg			
13	f/c	53	15 yrs			or/G,oc	pos	NA	14	neg	pos	neg	neg	pos	neg			

14	m/c	66	6 yrs			or/C,oc,n-ph	pos	pos	12	neg	neg	pos	neg	neg	neg			
15	m/c	42	5 yrs	y/G			pos	neg	12	neg	pos	neg	neg	pos	neg			
16	f/c	74	6 yrs	y /C			pos	neg	11	pos	neg	neg	neg	neg	*pos*			+/+ IgA
17	f/c	82	6 yrs	y			pos	neg	21	neg	neg	neg	neg	neg	pos	Pos*	pos	-/-
18	f/c	64	5 yrs			or/E,o,n-ph	pos	neg	4	pos	neg	neg	neg	neg	pos	Pos*	pos	
19	f/c	55	12 yrs	y/G			pos	neg	18	neg	neg	neg	neg	neg	pos	Pos*	pos	
20	m/c	79	7 yrs	no lesions (inactive)			pos	neg	0	neg	neg	neg	neg	neg	neg			
21	m/c	26	5 yrs			or/C,sk,oc,n-ph	pos	pos	4	pos	neg	neg	pos	neg	neg			+/+ IgG
22	m/c	64	10 yrs	no lesions (inactive)			pos	neg	0	neg	neg	neg	neg	neg	*pos*			-/+ IgG&A
23	f/c	64	3 yrs	y /G			pos	neg	12	neg	neg	neg	neg	neg	neg			
24	f/a	68	5 yrs			or/C,sk,gen, oc,n-ph	pos	NA	18	pos	pos	pos	pos	pos	neg			
25	f/c	58	8 yrs	y/G			pos	neg	24	neg	neg	neg	neg	neg	neg	neg	neg	+/-
26	m/c	67	10 yrs			or/G,gen	pos	neg	8	neg	neg	Pos×	neg	neg	neg	Pos×	pos	
27	f/c	69	12 yrs			or/C,sk,oc	neg	pos	35	pos	neg	neg	pos	neg	*pos*			-/-
28	f/c	75	18			or/E,sk,oc	neg	NA	13	pos	neg	pos	neg	neg	pos∨			

			yrs														
29	m/a	69	16 yrs		y		neg	neg	0	neg	neg	neg	neg	neg	*pos*			-/+ IgA
30	f/c	71	20 yrs			or/G,oc	pos	neg	6	neg	neg	neg	neg	neg	*pos*			-/-
31	f/c	79	15 yrs			or/G,n-ph no lesions(inactive)	pos	neg	0	neg	neg	neg	neg	neg	*pos*			
32	f/c	69	8 yrs	y/C			pos	pos	30	pos	pos	neg	neg	pos	neg			
33	m/c	80	9 yrs			or/G,oc	neg	NA	6	pos	neg	pos	pos	neg	neg			
34	m/c	80	6 yrs	y/G			neg	pos	12	neg	neg	neg	neg	neg	pos	Pos*	pos	
35	m/c	78	13 yrs	y/C			pos	neg	26	neg	neg	neg	neg	neg	pos	Pos*	pos	-/+ IgG&A
36	f/c	64	13 yrs			or/G,oc,n-ph	pos	neg	15	neg	neg	neg	neg	neg	neg	neg	neg	
37	m/c	61	12 yrs			or/C,oc	pos	neg	17	neg	neg	neg	neg	neg	neg	neg	neg	
38	f/c	81	15 yrs			Or,oc,n-ph (oral inactive)	neg	neg	0	neg	neg	pos	neg	neg	neg			+/-
39	m/c	67	12 yrs			or/G,oc	pos	pos	12	neg	neg	Pos°	neg	neg	neg	neg	neg	
40	m/c	44	2 yrs			or/G,oc,n-ph	pos	neg	14	pos	pos	pos	pos	pos	pos∨			-/+ IgG&A
41	m/c	87	18 yrs			or/G,sk,peri	pos	pos	5	pos	pos	pos	pos	pos	pos∨			
42	m/c	87	4 yrs			sk,oc,n-ph,peri	pos	pos	0	neg	pos	neg	neg	pos	neg			

43	m/c	75	10 yrs			oc,n-ph	pos	pos	0	pos	neg	neg	neg	neg	neg			+/+ IgA
44	f/c	57	2 yrs	y/G			pos	pos	12	pos	neg	neg	neg	neg	neg			-/+ IgA
45	m/c	83	11 yrs		y		NA	neg	0	neg	neg	pos	neg	neg	pos∨			-/-
46	f/c	59	8 yrs			or/E, oc	neg	NA	8	neg	neg	neg	neg	neg	neg			
47	f/c	46	7 yrs		y		neg	neg	0	neg	neg	pos	neg	neg	neg			-/-
48	m/c	48	4 yrs	y/G			pos	neg	6	pos	neg	Pos^	neg	neg	Pos^	neg	neg	
49	m/c	77	9 yrs			or/G,sk,oc	pos	neg	2	pos	neg	pos	neg	neg	neg			-/-
50	f/c	58	7 yrs			or/G,sk,gen, n-ph	pos	neg	14	pos	pos	neg	pos	pos	*pos*			
51	f/c	47	15 yrs	y/G			pos	neg	9	neg	neg	neg	neg	neg	neg			
52	m/c	65	11 yrs	y/G			pos	pos	12	pos	neg	neg	pos	neg	neg			-/-
53	f/c	69	6 yrs			or/G,n-ph	pos	neg	3	neg	neg	neg	neg	neg	pos	Pos*	pos	+/-
54	f/c	61	13 yrs			or/G,oc	pos	neg	1	neg	pos	neg	neg	pos	neg			-/-
55	m/c	71	10 yrs	y no lesions(inactive)			pos	pos	0	neg	pos	neg	neg	neg	neg			-/+ IgG
56	f/c	69	14 yrs		y		neg	neg	0	neg	neg	Pos+	neg	neg	pos	Pos+	pos	+/-
57	f/c	49	6 yrs	y/G			pos	neg	10	pos	neg	neg	neg	neg	pos	neg	neg	-/+ IgG
58	m/c	68	3 yrs	y no lesions(inac			pos	pos	0	neg	neg	neg	neg	neg	neg			

				tive)/ G														
59	f/c	60	12 yrs	y/G			pos	pos	12	pos	neg	Pos^	Pos	pos	Pos^	neg	neg	-/-
60	m/c	70	3 yrs			or/C,gen,n-ph	pos	neg	19	pos	neg	pos	neg	neg	neg			
61	f/c	70	7yrs	y/G			pos	neg	18	neg	neg	neg	neg	neg	neg			
62	m/c	71	12 yrs		y		neg	neg	0	neg	neg	neg	neg	neg	pos	Pos*	pos	-/+ IgG
63	m/c	64	4 yrs			or/C,oc	pos	NA	23	neg	pos	neg	neg	pos	neg			
64	m/c	57	7 yrs		y		pos	NA	0	neg	neg	neg	neg	neg	neg	neg	neg	
65	f/c	70	3 yrs			or/G,oc,n-ph	pos	NA	12	neg	neg	neg	neg	neg	neg			
66	f/c	73	9 yrs	y/G			NA	pos	10	neg	neg	neg	neg	neg	neg			+/+ IgA
67	m/c	44	11 yrs			or/C,oc	pos	neg	7	pos	neg	Pos^	Pos	neg	Pos^	neg	neg	-/-
68	m/c	64	15 yrs			Or/G,oc	neg	NA	8	neg	neg	neg	neg	neg	*pos*			
69	f/c	63	7 yrs	y/C			pos	neg	10	pos	pos	pos	pos	pos	pos∨			
70	f/c	62	9 yrs	y/G			pos	pos	6	neg	neg	neg	neg	neg	neg			
71	f/c	83	5 yrs		y		pos	NA	0	neg	neg	neg	neg	neg	neg	neg	neg	
72	m/c	60	7 yrs			sk,oc,n-ph	pos	neg	0	pos	neg	neg	neg	neg	neg	neg	neg	-/-
73	f/c	79	19 yrs		y		neg	NA	0	neg	neg	pos	neg	neg	pos∨			
74	f/c	55	12 yrs		y		pos	neg	0	neg	pos	neg	neg	neg	neg			-/-
75	m/c	62	8 yrs		y		pos	neg	0	neg	pos	pos	neg	neg	neg			-/-

76	f/a	63	2 yrs		y		pos	neg	0	neg	pos	neg	neg	neg	neg			-/-
77	m/c	44	2 yrs		y		neg	neg	0	neg	pos	neg	neg	neg	*pos*			-/-
78	f/c	65	2 yrs			Or(no leasions) oc,n-ph	pos	neg	0	neg	pos	neg	neg	neg	neg			+/-
79	m/c	80	6 yrs			or/C,oc,gen, sk	pos	pos	26	neg	pos	neg	neg	pos	neg	pos		
80	m/c	60	3 yrs			or/C,oc,scalp	pos	pos	43	pos	pos	neg	neg	pos	neg	pos		
81	f/c	75	18 yrs			or/C,nas,scalp	pos	pos	12	pos	neg	pos	pos	neg	pos	neg		
82	m/c	89	3 yrs			or/C,oc	NA	neg	38	neg	pos	neg	neg	neg	neg	neg		
83	f/c	66	5 yrs			or/G,gen	pos	pos	29	neg	pos	neg	neg	neg	neg	neg		
84	f/c	54	2 yrs	y/G			pos	neg	28	neg	neg	neg	neg	neg	neg	neg		
85	m/c	70	5 yrs	y/G			pos	neg	25	pos	pos	pos	pos	neg	neg	neg		
86	m/c	84	10 yrs			or/C,oc,n-ph	NA	pos	73	pos	pos	neg	neg	neg	neg	pos		
87	f/alg	29	4 yrs			or/C,n-ph,lar	pos	pos	23	neg	pos	neg	neg	neg	neg	neg		
88	f/c	79	3 yrs			or/C,oc,nas	pos	neg	25	neg	pos	neg	neg	neg	pos	neg		
89	f/c	54	4 yrs			or/G,oc	pos	pos	28	neg	pos	neg	neg	neg	pos	pos		
90	f/c	70	3 yrs			or/G,oc,nas, gen	pos	neg	10	neg	neg	neg	neg	neg	neg	neg		
91	m/c	51	7 yrs			oc, sk, n-ph	neg	neg	0	neg	pos	neg	neg	neg	neg	neg		
92	m/c	88	5 yrs	y/C			pos	pos	37	neg	pos	neg	neg	neg	neg			

93	f/c	63	9 yrs	y/C			pos	pos	12		pos			pos				
94	f/c	31	12 yrs	y/G			pos	neg	6		pos			neg				
95	m/c	89	3 yrs			or/C,peri,sk	pos	pos	22		neg			neg				
96	f/c	67	2 yrs	y/C			pos	neg	15		pos			pos				
97	f/afr	46	3 yrs	y/C			pos	pos	42		pos			pos				
98	f/c	48	6 yrs	y/C			pos	pos	15		pos			neg				
99	f/c	52	1 yr	y/G			NA	pos	20		pos			neg				
100	f/c	70	2 yrs	y/G			NA	neg	5		pos			neg				
Total Study 1(a)	78			25	16	50	59/76 78% (2 NA)	23/65 35% (13 NA)	53/78 68%	28/78 36%		22/78 28%	11/78 14%		30/78 38%	9/20 45%		39 11/39 28% (Lam 332) 7/39 18% (IgG 4575) 8/39 20% (IgA

																		4575)
Total Study 2	100			33	16	51	76/94 81% (6 NA)	36/87 40% (13NA)	74/100 74%		36/100 36%			18/100 18%				

DIF (Direct immunofluorescence), IIF (Indirect immunofluorescence), ODSS (Oral disease severity score), OIDS (Ocular inflammatory disease score), S (serum), P (Parotid saliva), ws (Whole saliva), SC (Secretory component i.e SIgA), or (Oral), oc (Ocular), n-ph (Nasopharyngeal), gen (Genital), sk (Skin), peri (Perianal), nas (Nasal), lar (Laryngeal), pos (Positive), neg (Negative), NA (Not Available), yrs (Years).

^ Samples positive for IgA specific antibody against BP180-NC16a on ELISA in whole saliva and serum (those having parotids).

Underlined samples positive for IgA specific antibody against BP180-NC16a on ELISA in whole saliva (having parotid)

***Underlined samples* positive for IgA specific antibody against BP180-NC16a on ELISA in whole saliva (no parotids)**

*** samples positive for IgA in whole and parotid saliva**

+ samples positive for IgA in serum, whole and parotid saliva

ᵛsamples positive for IgA in serum and whole saliva (no parotids)

Appendix 4 Patient demographic, clinical data and ELISA results

PV Samples	Sex	Age	Duration since diagnosis	Mucosal	Muco-cutaneous	DIF	IIF	ODSS	ELISA Dsg3/Dsg1	IgG Serum Dsg3	IgA serum Dsg3	IgG ws Dsg3
1	f	72	3 yrs	y		pos	pos	37	pos/neg	pos	pos	pos
2	f	59	7 yrs		y	pos	pos	37	pos/pos	pos	pos	pos
3	m	60	6 yrs	y		pos	pos	8	neg/neg	neg	neg	neg
4	f	54	5 yrs	y		pos	pos	13	pos/neg	pos	pos	pos
5	f	28	3 yrs		y	pos	pos	29	pos/neg	pos	neg	pos
6	m	20	2 yrs	y		pos	neg	45	pos/neg	pos	neg	pos
7	m	50	2 yrs	y		pos	pos	25	pos/neg	pos	pos	pos
8	m	78	5 yrs		y	pos	pos	17	pos/pos	pos	neg	neg
9	f	58	10 yrs	y		pos	pos	0	neg/neg	neg	neg	neg
10	m	45	2 yrs	y		pos	neg	18	neg/neg	neg	neg	neg

11	m	95	22 yrs		y	pos	pos	11	pos/neg	pos	pos	pos
12	f	62	3 yrs		y	pos	pos	8	pos/neg	pos	pos	pos
13	f	61	3 yrs	y		pos	pos	7	pos/neg	pos	pos	pos
14	m	37	2 yrs	y		pos	pos	0	pos/neg	pos	pos	neg
15	f	62	10 yrs		y	pos	pos	17	pos/neg	pos	pos	pos
16	f	64	5 yrs		y	pos	pos	7	pos/neg	pos	pos	pos
17	f	66	4 yrs	y		pos	neg	0	neg/neg	neg	neg	neg
18	m	41	3 yrs	y		pos	pos	27	pos/neg	pos	pos	pos
19	f	35	3 yrs	y		pos	neg	0	neg/neg	neg	neg	neg
20	f	69	7 yrs	y		pos	neg	4	neg/neg	neg	neg	neg
21	m	16	5 yrs		y	pos	pos	57	pos/pos	pos	pos	pos
22	m	34	5 yrs	y		pos	pos	21	pos/neg	pos	pos	pos

23	f	37	6 yrs	y		pos	neg	6	pos/neg	pos	pos	neg
Total	23			15	8	23 (100 %)	17 (74%)	19 (83%)	17 (74%) / 3 (13%)	17 (74%)	14 (61%)	14 (61%)

DIF (Direct immunofluorescence), IIF (Indirect immunofluorescence), ODSS (Oral disease severity score), S (serum), ws (Whole saliva), pos (Positive), neg (Negative), NA (Not Available), yrs (Years).

Appendix 5 Oral disease severity score (Escudier et al., 2007):

Oral Disease Severity Score (Escudier 2007)

Site	Site Score	Activity Score (0-3)
Outer lips (1)		
Inner lips (1)		
R Buccal mucosa (1 or 2)		
L Buccal mucosa (1 or 2)		
Gingivae (1 each segment)		
Lower R		
Lower central		
Lower L		
Upper R		
Upper central		
Upper L		
Dorsum of tongue (1 or 2)		
R Ventral tongue (1)		
L Ventral tongue (1)		
Floor of mouth (1 or 2)		
Hard palate (1 or 2)		
Soft palate (1 or 2)		
Oropharynx (1 or 2)		
Total		

Total Score
Total Score = Site Score + Activity Score + Pain Score (1-10)

Site Score
0 if no lesion
For the buccal mucosa
1 if less than 50% of area affected
2 if greater than 50% of area affected
For the dorsum of tongue, floor of mouth, hard or soft palate or oropharynx
1 unilateral
2 bilateral

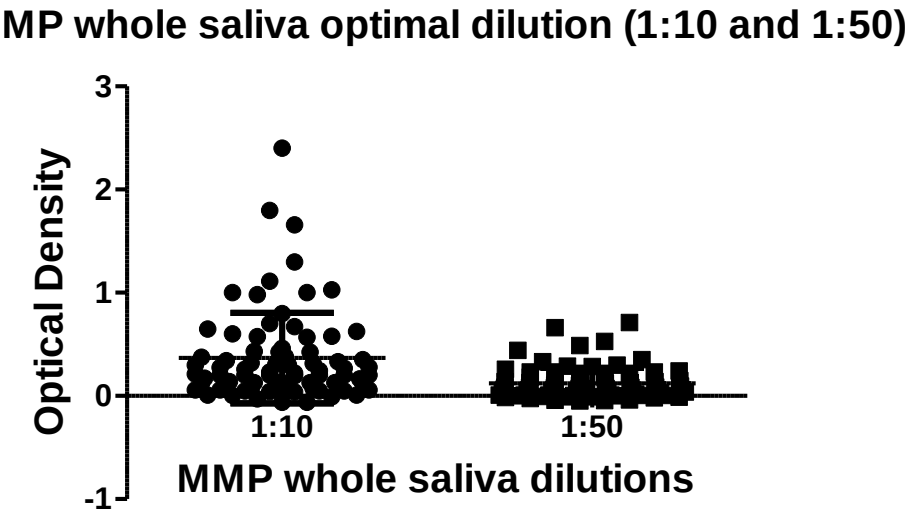
Activity Score
1 mild erythema (gingivae, papillae only or <3mm along margins)
2 marked erythema (full thickness gingivitis, extensive atrophy or oedema on non-keratinised mucosa)
3 ulceration at this site

Pain Score
Analogue scale from 0 (no discomfort) to 10 (the most severe pain they have encountered with this condition so far)
The patient is asked to provide a score reflecting their pain/discomfort as an average of the preceding week

Appendix 6 List of buffers and reagents used:

- PBS: pH 7.3, 137 mM sodium chloride, 2.7 mM potassium chloride, 8 mM disodium hydrogen phosphate, 2 mM sodium dihydrogen phosphate and distilled water (dH_2O) up to 1 L.
- PBS/ BSA/ Tween 20: PBS as above, BSA 0.5% and Tween 20 (Polyoxyethylenesorbitan monolaurate Tween 20).
- 1M Diethanolamine pH 9.8: magnesium chloride 0.1 g, 800 ml dH_2O, 97 ml diethanolamine, pH adjusted to 9.8 using concentrated 10M HCL and make up to 1 L with dH_2O. This was stored in the dark at 4°C.
- 3M NaOH pH 14: 200 ml dH_2O, 24 g sodium hydroxide.
- 1XTransfer buffer pH 8.3: 70% dH_2O, 20% methanol and 10% of 10X transfer buffer (250 mM Tris Base, 2.5 M glycine, 1% sodium dodecyl sulphate SDS).
- De-staining buffer: 20% methanol, 10% acetic acid 70 ml dH_2O
- Washing solution: PBS/ Tween 20, PBS as described and 0.05% Polyoxyethylenesorbitan monolaurate Tween 20.
- Carbonate/ bicarbonate buffer pH 9.6 (100 mM): 3.03 g sodium carbonate, 6.0 g sodium bicarbonate, adjust pH 9.6 using concentrated HCL and make up to 1 L with dH_2O.
- Phosphatase substrate (4-nitrophenyl phosphate disodium salt hexa-hydrate) tablet form (N2765, SIGMA -ALDRICH)
- Recombinant human integrin alpha 6 beta 4 ($\alpha6\beta4$) 50 µg (5497-A6-050 R&D systems)

Appendix 7 Whole saliva optimal dilution (1:10 vs 1:50)



Appendix 8 α6β4 optimal coating concentration

Plate coated with α6β4 at 3 concentrations (**0.1**, **0.5**, **1 µg/ml**) . Anti-α6 antibody at different dilutions (1:250.1:500,1:100)

Plate coated with α6β4 at 3 concentrations (**0.1**, **0.5**, **1 µg/ml**). Anti-β4 antibody at different dilutions (1:250.1:500,1:100)

	1:250	**1:500**	**1:1000**	**No antibody**	**1:250**	**1:500**	**1:1000**	**No antibody**
0.1 µg/ml	1.03087	0.825272	0.555758	0.158264	2.54422	1.97947	2.27619	0.187914
0.1 µg/ml	0.937113	0.735883	0.517709	0.138291	2.4464	1.86013	2.12587	0.135618
0.5 µg/ml	2.62496	2.21692	1.34453	0.153824	3.05953	3.15644	3.07726	0.15152
0.5 µg/ml	2.54663	2.20038	1.28436	0.158267	2.94193	2.90414	2.92897	0.154988
1 µg/ml	2.74689	2.45965	1.31486	0.153861	3.04792	3.01316	2.96574	0.148377
1 µg/ml	2.53992	2.14198	1.32565	0.132487	2.61676	2.71896	2.71019	0.124116
No protein coating	0.177948	0.171477	0.167917	0.167992	0.164461	0.1761	0.187644	0.182025
No protein coating	0.200976	0.172364	0.165904	0.162235	0.261291	0.158929	0.16165	0.17684

The first row indicates antibody dilutions. The first column indicates protein concentrations. The rest of the table shows optical density results (OD 405 nm).

- Coating overnight 4°C (α6β4 at different concentrations in Carbonate Bicarbonate buffer Ph 9.6)
- Blocking with PBS/BSA/T20 37°C 1 hr
- Antibody diluted in PBS/BSA/T20 at different dilutions and incubated for 2 hrs 37°C (both anti-α6 and anti-β4)
- Anti-species Conjugate added diluted at 1:1000 in PBS/BSA/T20 alkaline phosphatase linked 1 hr 37°C
- Substrate added developed for 30 minutes 37°C
- Reaction stopped by 3MNaOH and plate read at 405 nm

Appendix 9 IgA serum and saliva optimization for PV Dsg 3 plates

- **Plate 1 Dsg3 pre-coated plates: Sample incubation 2 hrs, 1:500 anti-human IgA conjugate (OD at 405 nm) (PV, DC -MMP,LP-, HC, Positive control, Negative control)**

A): Serum

1.9	0.99	1.5
2.25	0.78	0.9
3	3	1.7
2.25	1.7	1.3
1.	0.8	1.2
1.5	1.8	1.8
2.3	1.2	1.7
1.25	1.3	0.7

B): Saliva

2	0.5	0.6
1.1	0.8	1.3
1.4	1.1	0.7
1.2	1	0.8
1.3	0.7	1.1
1	0.9	0.8
2.6	1.9	0.8
2	1.7	0.6

- **Plate 2 Dsg3 pre-coated plates: Sample incubation 1 hr, 1:1000 anti-human IgA conjugate (OD at 405 nm) (PV, DC -MMP,LP-, HC, Positive control, Negative control)**

A): Serum

1.5	0.64	0.12
0.8	0.73	0.2
0.7	0.88	0.22
0.9	0.52	0.18
0.54	0.24	0.078
0.34	0.12	0.17
0.33	0.11	0.9
0.78	0.9	0.18

B): Saliva

0.29	0.15	0.098
0.24	0.12	0.18
0.23	0.3	0.13
0.27	0.22	0.22
0.3	0.23	0.15
0.22	0.22	0.16
0.25	0.27	0.7
0.22	0.29	0.2

9 References

9 References

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